# REMARKS

Applicants respectfully request entry of the Sequence Listing into the above-identified application. A computer readable form of the Sequence Listing is also enclosed. Applicants state that the content of the sequence listing information recorded in computer readable form is identical to the paper version included herewith. The enclosed Sequence Listing replaces the sequence listing as filed June 26, 2009.

The revision to the sequence listing replaces Ser with Ala in the 12 position of Seq Id Nos. 10-18. Applicants submit that the revision would be recognized as an editorial correction. Note that page 5, lines 9-11 of the present specification indicate that Seq Id Nos. 10-18 are human origin sequences. Ellison et al. discloses the nucleotide sequence for TAN-1 in fig. 2 on page 651. Bash et al. is provided to confirm the correspondence between TAN-1 and human notch I in the right column of page 2803, lines 17-22.

In view of the above, early issuance of a notice of allowance is solicited. Any questions regarding this communication can be directed to the undersigned attorney, Douglas P. Mueller, Reg. No. 30,300 at (612) 455-3804.
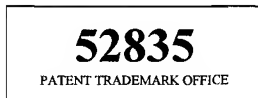
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TAN-1, the Human Homolog of the Drosophila *Notch* Gene, Is Broken by Chromosomal Translocations in T Lymphoblastic Neoplasms

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Summary

Previously we described joining of DNA in the β T cell receptor gene to DNA of an uncharacterized locus in a t(7;9)(q34;q34.3) chromosomal translocation from a case of human T lymphoblastic leukemia (T-ALL). We now show that the locus on chromosome 9 contains a gene highly homologous to the Drosophila gene *Notch*. Transcripts of the human gene, for which we propose the name *TAN-1*, and its murine counterpart are present in many normal human fetal and adult mouse tissues, but are most abundant in lymphoid tissues. In t(7;9)(q34;q34.3) translocations from three cases of T-ALL, the breakpoints occur within 100 bp of an intron in *TAN-1*, resulting in truncation of TAN-1 transcripts. These observations suggest that *TAN-1* may be important for normal lymphocyte function and that alteration of *TAN-1* may play a role in the pathogenesis of some T cell neoplasms.

Introduction

Evidence is accumulating for many tumors that chromosomal translocations play a direct role in neoplastic transformation and tumor progression. Genes located at or near the breakpoints of several common translocations in various forms of leukemia and lymphoma have been molecularly cloned, and it is apparent that in some cases these genes are proto-oncogenes related to previously identified genes in transforming retroviruses. In other cases, the genes located at translocation breakpoints have no known counterparts within the genomes of oncogenic viruses, nor have they yet been shown to possess transforming properties after transfection into cultured cells or insertion into transgenic animals. Nevertheless, virtually all genes lying at sites of translocation breakpoints are believed to function in some aspect of normal cell growth or differentiation. Alterations in the structure and/or expression as a result of the translocation is a frequent finding, and it is in this manner that the presence of the translocation presumably contributes to the process of neoplastic transformation. Exactly how these translocated genes participate in

the pathogenesis of these neoplasms is, however, still poorly understood.

Cytogenetic studies have shown that chromosome 7 band q34-35, which contains the gene for the β T cell receptor (*TCRβ*), is a common site for translocation in T cell neoplasms (Raimondi et al., 1987). Molecular cloning has confirmed involvement of the *TCRβ* gene in at least five different translocations showing cytogenetic breakpoints at 7q34-35 (Reynolds et al., 1987; Tycko et al., 1989; Russo et al., 1988; Mellentin et al., 1989; Tycko et al., 1991). Recently, we described the cloning of a breakpoint from one of these translocations, t(7;9)(q34;q34.3), found in a case of acute T cell lymphoblastic leukemia (T-ALL). In this case, the translocation involved the *TCRβ* gene and a second locus on chromosome 9, which was originally referred to as *TCL-3*. DNA in this locus was shown to be transcribed at a high level in a cell line carrying the translocation.

We report here the structural analysis of an 8.3 kb transcript derived from this locus on chromosome 9 in normal human cells. Nucleotide sequence analysis of cDNAs representing in aggregate nearly the full-length transcript produced from the locus indicates that it encodes a protein highly homologous to the Drosophila *Notch* gene product, an integral membrane protein involved in the developmental determination of embryonic cell fates (Artavanis-Tsakonas et al., 1983; Xu et al., 1990). The human gene also shows varying degrees of homology to other *Notch*-related genes, including *Xotch*, the Xenopus homolog of *Notch* (Coffman et al., 1990), and two genes of Caenorhabditis elegans, *lin-12* and *glp-1* (Yochem et al., 1988; Yochem and Greenwald, 1989). Based on this homology, we propose that the human gene be renamed *TAN-1*, an acronym for translocation-associated *Notch* homolog.

Our work demonstrates that *TAN-1* is expressed in a variety of normal human fetal tissues, as is a related murine gene in many adult mouse tissues. However, it is relatively overexpressed in lymphoid tissues from both mice and humans. Studies of *TAN-1* in three cases of human T-ALL containing t(7;9)(q34;q34.3) translocations show that the breakpoints are tightly clustered within the *TAN-1* locus and that the effect of translocation is the production of truncated versions of *TAN-1* mRNA. These results suggest that the human *Notch* homolog functions in normal lymphoid development but, in rearranged form, may contribute to transformation or progression in some T cell neoplasms.

Results

Cloning of cDNA and Sequence Analysis of *TAN-1*

The top portion of Figure 1 shows a schematic drawing of DNA surrounding the t(7;9)(q34;q34.3) breakpoint cloned from the SUP-T1 cell line, which had been derived from a patient whose tumor carried the translocation (Reynolds et al., 1987). We had previously shown that the genomic probe PS 1.3, a chromosome 9 probe isolated from DNA

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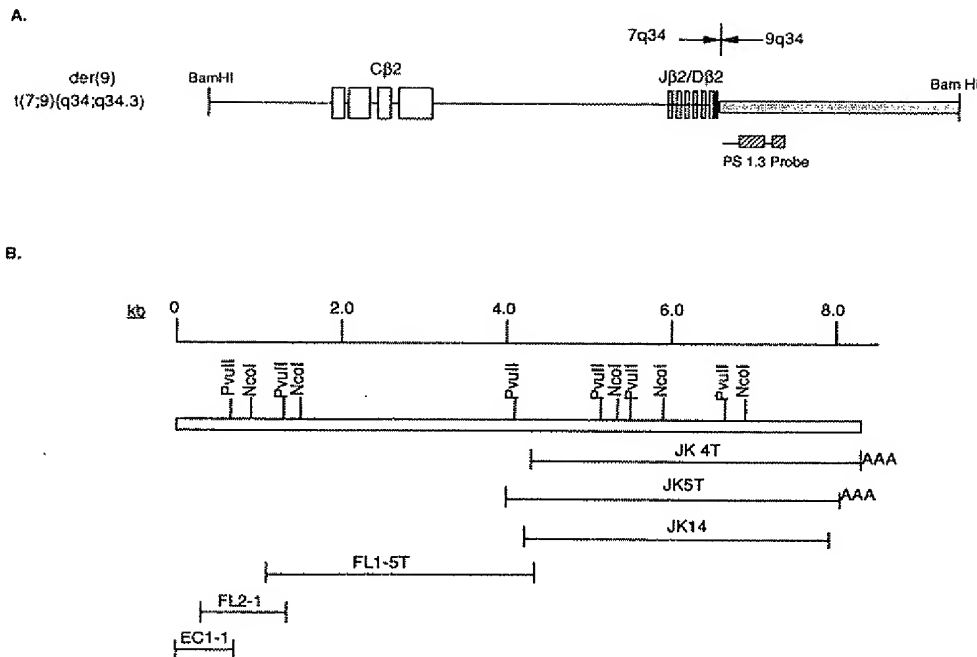
A.

B.

Figure 1. Schematic Representations of the t(7;9)(q34;q34.3) Breakpoint DNA and the *TAN-1* cDNA

(A) Diagram of DNA surrounding the der(9) breakpoint of t(7;9)(q34;q34.3) previously cloned from case 1. Shown is the structure of a 12 kb BamHI fragment containing DNA sequences of *TCRβ* (from chromosome 7, left) joined to DNA of *TAN-1* (from chromosome 9, right) at the breakpoint of the t(7;9) from case 1, as described by Reynolds et al. (1987). The figures are oriented with respect to the direction of normal transcription of *TAN-1* from 5′ (left) to 3′ (right). Below the diagram of the der(9) breakpoint is the PS 1.3 probe, which was subcloned from the DNA above and used to isolate cDNA clones JK4T, 5T, and 14. Exon sequences of PS 1.3 are represented by cross-hatched boxes.

(B) Restriction map of composite *TAN-1* cDNA sequences and schematic diagram of overlapping *TAN-1* cDNA clones used for sequence analysis. Shown below the kilobase scale is a restriction map of the composite cDNA representing a near full-length *TAN-1* transcript. The relative positions of the individual cDNA clones are shown beneath this map. The JK clones were isolated from an oligo(dT)-primed cDNA library of Jurkat cell line RNA. The FL clones were isolated from cDNA libraries of FL18 cell line RNA primed with specific oligonucleotides. The clone EC1-1 was isolated from a randomly primed cDNA library of cultured human umbilical vein endothelial cell RNA (Ginsburg et al., 1985).

adjacent to the translocation breakpoint on the derivative chromosome 9 product, der(9), detected a major transcript of 4.5 kb in the SUP-T1 cell line. In addition, a much less abundant transcript of about 8.5 kb was detected in several T and B cell lines that lacked the translocation. To characterize the structure of the transcript produced from untranslocated chromosome 9 DNA, a phage library of oligo(dT)-primed cDNA was prepared from poly(A)$^+$ RNA (a gift from Gerald Crabtree, Stanford University) extracted from Jurkat cells, a mature T cell line, and screened with the PS 1.3 probe. Three overlapping 4.0 kb cDNA clones, JK4T, 5T, and 14, were isolated from the library and sequenced. The positions of these clones are shown schematically in Figure 1. To isolate a cDNA clone containing sequences 5′ of the three Jurkat clones, a specific cDNA library was generated by reverse transcription of poly(A)$^+$ RNA obtained from the human B lymphoma cell line FL18 (Doi et al., 1987) and primed with an oligonucleotide containing sequence lying near the 5′ end of clone JK5T. The longest cDNA resulting from this procedure, clone FL1-5T, extended 3.5 kb (Figure 1). Using sequence determined at the 5′ end of this cDNA, another round of oligonucleotide-primed cDNA cloning was carried out to isolate clone FL2-1, which was found to extend 1.0 kb 5′ beyond the end

of FL1-5T. An additional 270 bp of sequence were obtained from a cDNA library constructed by random priming of human umbilical vein endothelial cell poly(A)$^+$ RNA (the gift of Tucker Collins and Stuart Orkin, Harvard Medical School) and screened with a probe generated from the 5′ end of the cDNA clone FL2-1.

Nucleotide sequences determined from each of these clones were assembled into a continuous sequence 8.3 kb in length. These sequences were compared with those listed in the GenBank using the University of Wisconsin Genetics Computer Group program WORDSEARCH (Devereux et al., 1984). The highest scoring match was with *Notch*.

The *TAN-1* cDNA contains an open reading frame of 2555 amino acids (Figures 2 and 3). The N-terminal halves of the putative *TAN-1* protein and the *Notch* protein are both comprised of 36 tandemly iterated units that have been termed the epidermal growth factor (EGF) cysteine repeats. Each repeat consists of a stretch of about 40 amino acids in which cysteine residues and certain other amino acids are highly conserved. These units occur once in the mature EGF peptide and several times in various cell surface and secreted proteins (Figure 4A). Within this region, the amino acid sequences of *TAN-1* and *Notch* are
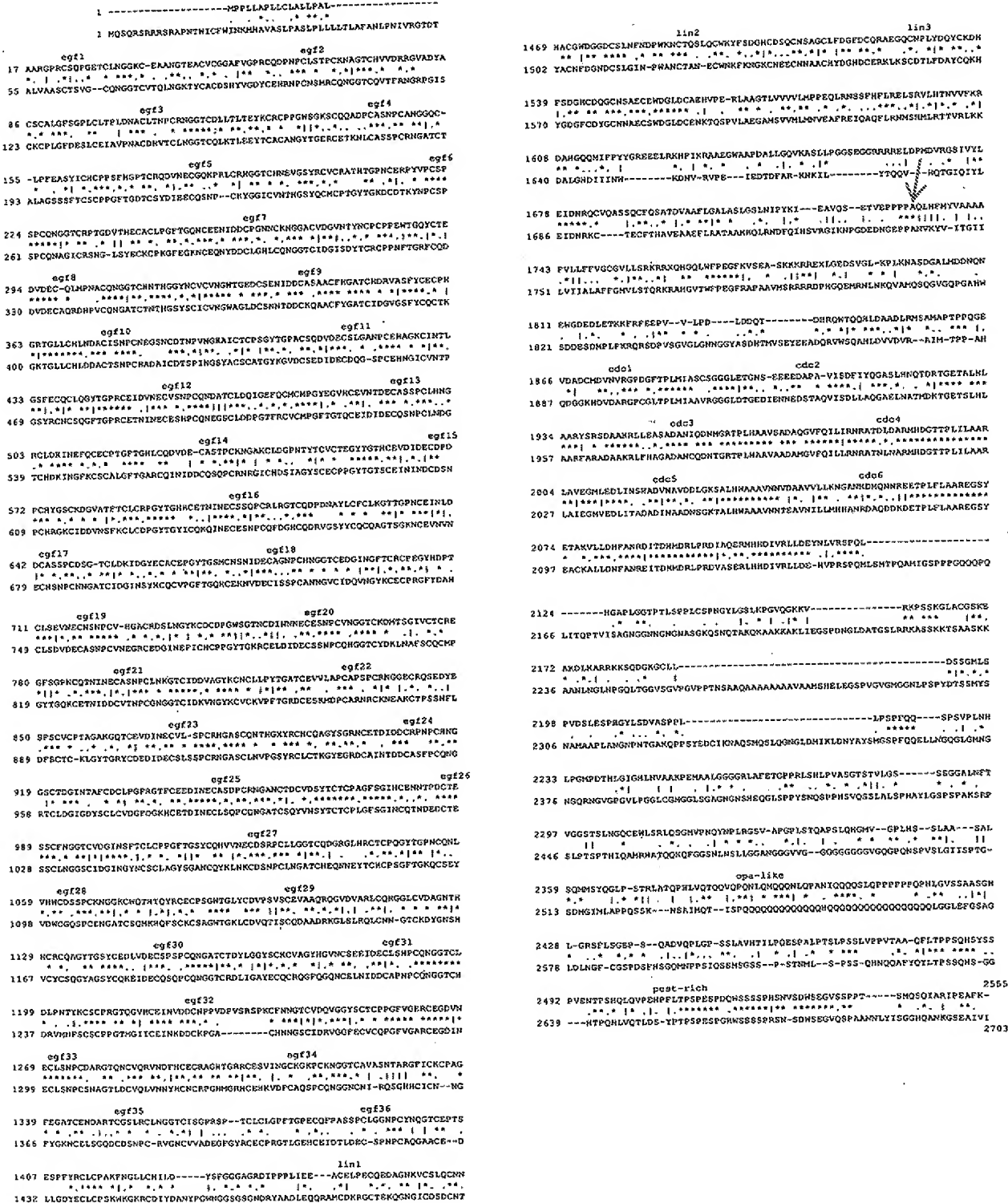
Figure 2. The Deduced Amino Acid Sequences for *TAN-1* and *Notch*

The *TAN-1* sequence is shown above the *Notch* sequence. The sequences were aligned with the LOCAL program for amino acid sequence comparison (Smith et al., 1985). Asterisks indicate identical residues; vertical lines and dots indicate first and second degree conservative differences, respectively; dashes indicate gaps introduced to maximize the amount of alignable sequence. Labels have been placed at the beginning of sequence motifs.
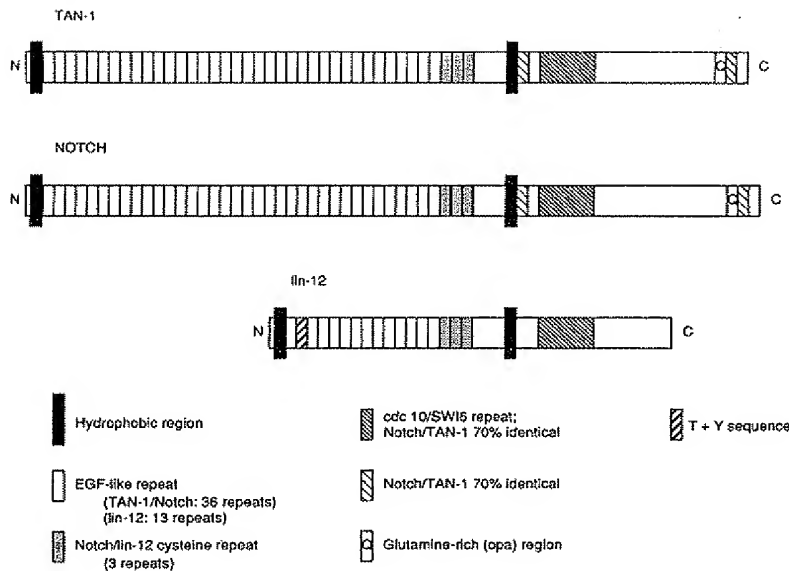
Figure 3. Schematic Alignment of Homologous Domains in the Amino Acid Sequences for the Putative *Notch*, *TAN-1*, and *lin-12* Proteins

Homologous domains within the three protein sequences are shown and identified individually below. The hydrophobic domain at the N-terminus of all three protein sequences is the presumed signal peptide, while the hydrophobic region in the middle of the three proteins is the supposed transmembrane domain.

over 50% identical (and 67% similar taking into consideration conservative changes) with only one significant gap of 8 amino acids distinguishing the two proteins.

Immediately on the carboxy side of the EGF cysteine repeats is another iterated cysteine-rich motif, which has been referred to as the *Notch/lin-12* cysteine repeat because it is shared by *Notch* and the two C. elegans genes, *lin-12* and *glp-1* (Yochem et al., 1988) (Figure 4B). The *Notch/lin-12* repeated element is about 40 amino acids in length and occurs three times in each of the genes in the *Notch/lin-12* group (including *Notch*, *Xotch*, *lin-12*, *glp-1*, and *TAN-1*). *TAN-1* and *Notch* show a degree of homology here similar to that within the EGF-like repeats, but each is only about 30% identical with *lin-12* or *glp-1* over this region.

C-terminal to the *Notch/lin-12* repeat is a stretch of about 110 amino acids in *TAN-1* and 90 amino acids in *Notch* that share several small regions of sequence identity. This part of the two genes is followed by a stretch of 19 and 21 hydrophobic amino acids in *TAN-1* and *Notch*, respectively, which is followed in turn by three basic amino acids in both proteins. Sequences similar to this are characteristic of membrane-spanning domains of many transmembrane proteins (Ullrich et al., 1985).

The region of highest homology between *TAN-1* and *Notch* begins about 120 amino acids on the C-terminal side of the transmembrane domain. Over a stretch of 245 amino acids, the peptide sequences of the two genes are more than 70% identical and 83% similar. Most of this region contains a sequence motif repeated six times in all members of the *Notch/lin-12* gene family (Figure 4C). A duplicated version of this motif was first identified in two yeast cell cycle control genes: *cdc10*, which is required at the start of the cell cycle in Schizosaccharomyces pombe, and *SWI6*, a trans-activator of cell cycle–controlled transcription in Saccharomyces cerevisiae (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989). Each of

these so-called *cdc10/SWI6* repeats is 35 amino acids long, and the repeated sequences within or between genes are loosely conserved, showing about 35% sequence homology between *TAN-1* and *Notch*.

From the last *cdc10/SWI6* repeat to the C-terminus of the coding region, the homology between *TAN-1* and *Notch* is lower than in the rest of the sequences. *TAN-1* has about 20% fewer amino acids in this region than does *Notch*, although *TAN-1* and *Xotch* are over 80% identical in their 500 C-terminal amino acids. There are, however, two sequence motifs in this region that are relatively conserved between *TAN-1* and *Notch*. The first is a stretch of nearly pure (30 of 31 positions) glutamine amino acids in *Notch* — a motif that is called the opa repeat and has been suggested to affect protein stability (Wharton et al., 1985). The second conserved region, about 50 amino acids from the C-terminus of both *TAN-1* and *Notch*, consists of amino acid sequences enriched for proline (P), glutamic acid (E), serine (S), and threonine (T). This motif has consequently been referred to in other proteins as a PEST sequence. In addition, other amino acids besides the four characteristic PEST amino acids are conserved between *TAN-1* and *Notch* in this part of the two sequences.

Expression of *TAN-1* in Human Fetal and Adult Mouse Tissues

To examine the expression of the *TAN-1* gene in developing human tissues, we prepared total RNA from individual tissues and organs derived from 18–20 week gestational age, anatomically normal human fetuses. Expression was assayed both by Northern blot analysis using various *TAN-1*–derived probes (data not shown) and by RNAase protection studies with a probe generated from the region of the cDNA containing the most 3′ group of EGF-like repeats (Figure 5A). Possible differences in the amounts of RNA loaded on gels for Northern blot analysis were controlled for by stripping and reprobing the blot with a

A

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Consensus        C              C  N  G  C          C  C  GF G    C
TAN-1(r35)    CLGGNP    CMNCCTCEPTSESPFYHCLCRAKFNCLLCHI
TAN-1(r36)    CGSLR     CLNGSTCLSGPRSPT   CICLGHETCFECQF
Notch(r26)    CTESS     CLNGSFIDGINGYN    CSCLNGYSCANCQY
Notch(r14)    COSQP     CRNRGICHDSIAGYS   CECPHCYTCTSCB
C.ele.lin-12  CLENP     CSNGGVCROHRESFS   CDCPECFYCNGCEQ
Factor IX-hu  CESNP     CLNGGSCKDDINSYE   CWCPFGFRSKKCEL
Factor X-hu   CETSP     CONCGCKDGLGEYT    CTCLLGFECKNCEL
EGF-mouse     CPSSYDGYCLNGGVCMHIESDLSYTCNCVIGYSCDRCQT
TGF-rat       CPDSHTQYCFHGT CRFLVQEEKPACVCHSGYVGVRCEH
tPA-hu        CSEPR     CFNGGTCQQALYFSDFVCQCPEGFAGKCCEI
mLHRc         CQPGS     CNGRSPCVETINNHT   CICPAGYYCPCQQY
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B

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Consensus C     .         C     F  NG  CD    CN    C  DG  DC
TAN-1     CELPE         CQEDAGNKVCSLQCNNGACGWDGGCSLNFNDPWKN
Notch     CDKRG         CTEKQGNGICISDCMTYACNFDGNCDCSLGIN.PWAN
lin-12    CEKRK         CSERANDGNCLALCNYAACKFDGCDCSGKRE.PFSK

          CTQSLQ        CWKYFSDGHCDSQCNSAGCLFDGFDCQRAEGQ
          CTA.NE        CWNKFKNGKCNEECNNAACHYDGNDCERKLKS
          CRYGNM        CAGFFANGVCNQACNNEFCLYDGMDCLPAVVR

          CNPLYDQYCKDHFSDGKCBQGCNSAECEWDGLDCAEHV
          CDTLFDAYCDKHYGDGFCDYGCNNAECSWDGLDCENKT
          CPVKIREHCASRFANGICDPECNFNCCGFDGSDCDNET
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C

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TAN-1   NVRGPDGFTPLMIASCSGGGLETGNSEE..EEDAPAVISDFIYQGASL
        :.)II.I:IIIIII...II)I:II:. I  I:..r.IIIII:: II).I
Notch   DARGPCGLTPLMIAAVRGGGLDTGEDIENNEDSTAQVISDLLAQGABL

        HNQTDRTGETALHLAARYSRSDAAKRLLEASAD
        :.  I:III).IIIIII:.I.PIIIIII:.I:II
        NATMDKTGETSLHLAARFARADAAKRLFHAGAD

        ANIQONMGRTPLHAAVSADAQGVFQILIRNRATD
        II.III IIIIIIIII.III IIIIII:IIII:II
        ANCQONTGRTPLHAAVAADAMGVFQILLRNRATN

        LDARMHDGTTPLILAARLAVEGMLEDLINSHAD
        I:IIIIIIIIIIIIIII::III:IIII...II
        LNARMHDGTTPLILAARLAIEGMVEDLITAOAD

        VNAVDDLGKSALHWAAAVNNVDAAVVLLKNGAN
        II.I: II.IIICIIIII:.:. :II.: II
        RNAADNSGKTALHWAAAVNNTEAVNILLMHHAN

        KDMQNNREETPLFLAAREGSYETAKVLLDHFAN
        :I I:::::IIIIIIIIIIII..i.III:III
        RDAQDDKDETPLFLAAREGSYEACKALLDNFAN

cdc10   DVPIDELGHAALGWAAAVAKMPLLQALIHHGAN

        LNGQDKNGDTALHIAARIGNKNIVEVLHQAGAS

SWI6    NIPVOEHGNTPLHWLTSIANLELVKHLVEHGSN

        LNAQDSNGDTCLNIAARIGNISIVDALLDYGAD

Consensus  N  D  G T LH AAR          L    A
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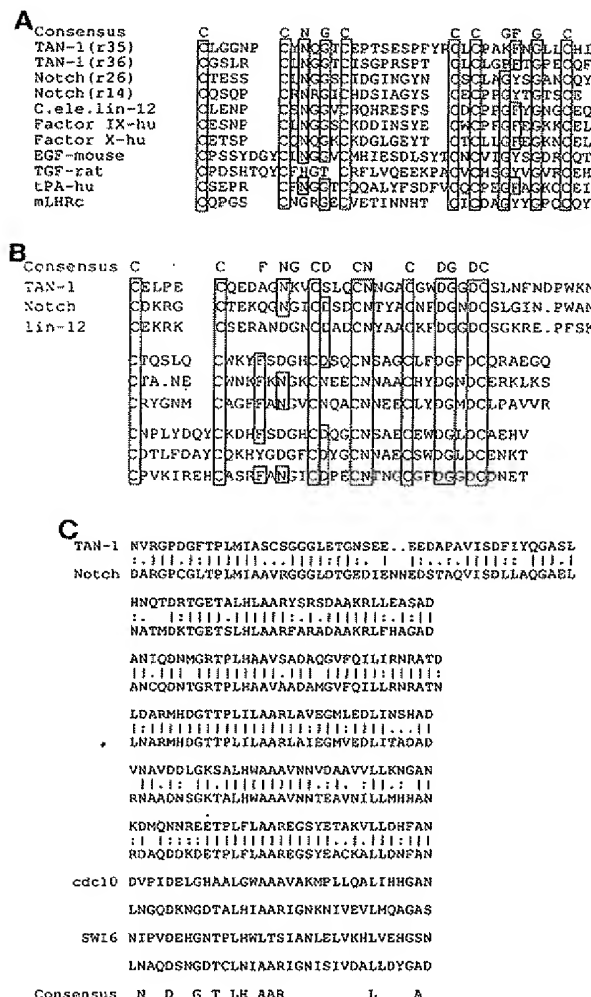
Figure 4. Comparison of Amino Acid Sequences of Various Homologous Domains Common to the *TAN-1/Notch/lin-12* Protein Group

(A) Comparison of the EGF-like cysteine repeats. Two EGF-like repeats from both *TAN-1* and *Notch* are compared with those of other repeat-containing proteins, including *lin-12*, human clotting factors IX and X, murine EGF, rat transforming growth factor, and the mouse lymphoid homing receptor. The most highly conserved amino acids are boxed, although other amino acids are also partially conserved.

(B) Comparison of the *TAN-1/Notch/lin-12* cysteine-rich repeats. The sequence of amino acids in the three repeats present in each of the three proteins is illustrated. Only one other known protein, *glp-1* from C. elegans, contains this repeated motif, but like *lin-12* it is less homologous to *Notch* than is *TAN-1*. Conserved amino acids are boxed.

(C) Comparison of the amino acid sequences of the *cdc10/SWI6* repeat in *TAN-1*, *Notch*, *cdc10*, and *SWI6*. Vertical lines between the sequences indicate identical amino acids; two dots, conservative differences; one dot, less conservative differences.

probe from the human γ actin gene (Gunning et al., 1983). Actin RNA was also used as a control for amounts of RNA assayed by the RNAase protection method. Results from the two analyses were concordant and showed that *TAN-1* is expressed nearly ubiquitously in the 18 human fetal tissues examined, although the amount of transcript varies more than 5-fold between various tissues. The fetal tissues containing the highest level of *TAN-1* transcript were, in descending order, spleen, brain stem, and lung.

Since data on larval development in the fruit fly suggest that expression of *Notch* correlates with mitotic activity in different tissues (Kidd et al., 1989), we attempted to determine whether the relative levels of expression in a variety of adult tissues might differ from one another compared with levels in fetal tissues, all of which might be expected to contain high numbers of mitotic cells. Owing to the difficulty in obtaining fresh adult human tissues with reliably intact RNA, we prepared total RNA from a variety of adult mouse tissues and examined *TAN-1* expression by Northern blot or RNAase protection analysis. As a probe for Northern analyses of murine tissues, we used human *TAN-1* probes, since Southern blot analyses even at high stringency revealed that all human *TAN-1* probes cross-hybridize with single copy mouse sequences (data not shown). For RNAase protection studies, a probe was constructed from cDNA synthesized from poly(A)$^+$ mouse spleen RNA by reverse transcription using oligonucleotide primer complementary to the sequence of the *cdc10/SWI6* region of *TAN-1*, followed by polymerase chain reaction (PCR) amplification of the single-stranded cDNA using two other oligonucleotide primers complementary to sequences within the *cdc10/SWI6* region of *TAN-1* and 5′ to the sequence of the primer used for reverse transcription.

Results of the RNAase protection analysis on RNA from various adult mouse tissues is shown in Figure 5B. Since no mouse actin probe was available to us, the amount and integrity of the RNA in the samples were verified on a separate ethidium bromide-stained gel (data not shown). Contrary to expectations, the expression profile of the *TAN-1*–homologous gene in adult mouse tissues was nearly identical to that in fetal human tissues. The transcript was present in every tissue tested except the small intestine. In addition, the relative levels of the *TAN-1*–homologous murine transcript were the same in adult mouse tissues as was *TAN-1* transcript in human fetal tissues. Lymphoid tissues, central nervous system, and lung showed the highest level of expression. There was, however, a reproducible reversal of the ratio in the expression of *TAN-1* between the spleen and the thymus of adult mouse tissues as compared with the human fetal ratio. This reversed ratio was also found with adult human tissues.

Analysis of Breakpoints in Chromosomal Translocations Involving *TAN-1* and *TCRβ*

To estimate the frequency of *TAN-1* rearrangement in T lymphoblastic neoplasms, DNA was prepared from a total of 40 T cell lymphoblastic lymphomas and leukemias, digested with multiple restriction enzymes, and screened by Southern blot analysis with the PS 1.3 genomic chromosome 9 DNA probe. Out of the 40 cases studied, four leukemias each showed one rearranged band analyzed with two or more enzymes. Involvement of the *TCRβ* locus in these rearrangements was examined by stripping the blots and rehybridizing with a probe for this locus. In two cases, rearranged bands detected with a probe for the first *TCRβ* joining region (J$_\beta$1) were superimposable with the
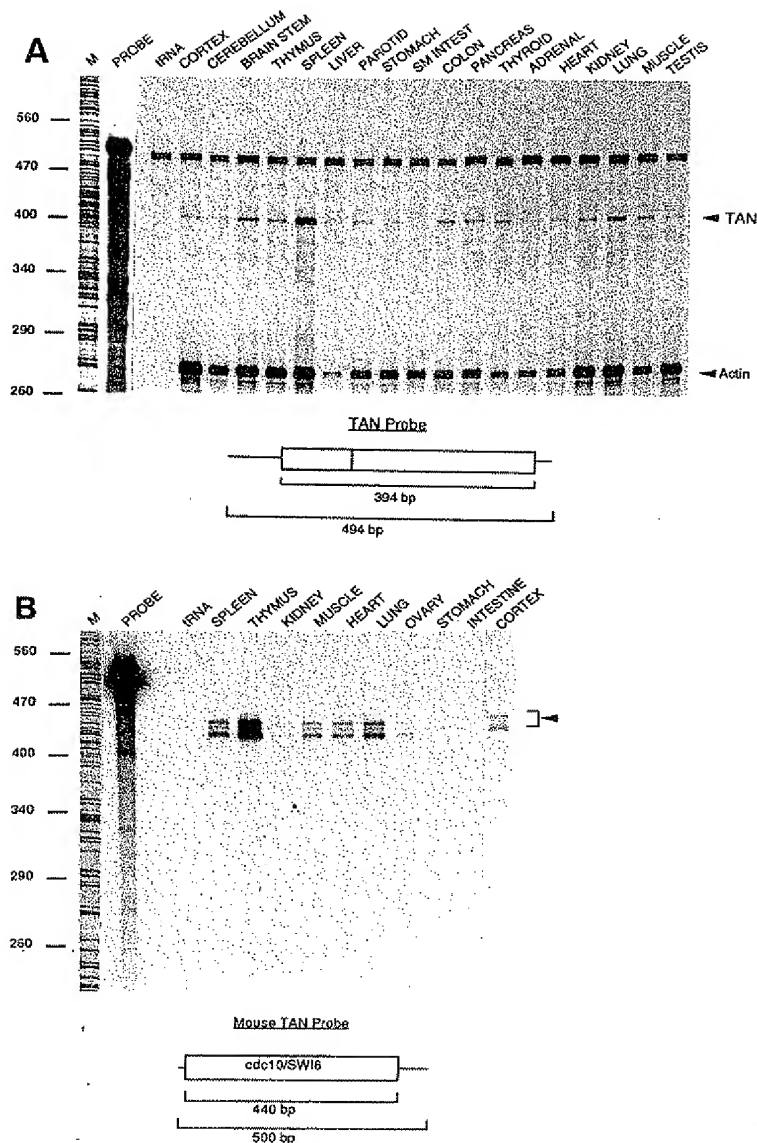
Figure 5. RNAase Protection Analysis of *TAN-1* RNA Transcripts in Human Fetal Tissues and *TAN-1*–Homologous Transcripts in Adult Mouse Tissues

(A) Expression of *TAN-1* RNA in human fetal tissues. In each analysis, RNAase protection was performed using 10 μg of total RNA extracted from tissues of 10–20 week gestational age, anatomically normal human fetuses. Two single-stranded RNA probes were hybridized simultaneously to each RNA sample. The *TAN-1* probe, shown schematically at the bottom, produces a protected band of 394 bp (the position of which is indicated by the top arrowhead at the right) when hybridized to normal *TAN-1* transcripts. A human γ actin probe, included to control for the amount and integrity of the RNA, produces a protected band of 265 bp (bottom arrowhead at right) with normal human RNA. Hybridization against yeast transfer RNA (tRNA) was included as a negative control. The lane labeled PROBE shows the position of unhybridized probe RNA. Size markers (M) consisted of ^{35}S-labeled DNA.

(B) Detection of *TAN-1*–homologous RNA transcripts in adult mouse tissues. RNAase protection analysis was performed using 10 μg of total RNA extracted from tissues of adult B57 mice. A DNA fragment containing sequences from the mouse homolog of *TAN-1* was prepared by carrying out PCR on single-stranded cDNA generated from mouse spleen RNA using oligonucleotides corresponding to human *TAN-1* sequences, as described in the text. A fragment was subcloned from the PCR product and transcribed to generate a 500 bp single-stranded RNA probe, which yielded a protected 440 bp fragment (shown schematically at the bottom) when hybridized to mouse *TAN-1*–homologous sequences. Three bands repeatedly produced by hybridization to mouse *TAN-1*–homologous transcripts probably result from mismatches between the mouse *TAN-1* transcript and the human oligonucleotides incorporated into the probe fragment. The labels PROBE, tRNA, and M have the same meanings as in (A). The amount and integrity of the mouse RNA was verified on separate ethidium bromide–stained gels (data not shown).

bands detected with the PS 1.3 probe (Figure 6). Karyotype analyses from one of these cases (MM) showed a t(7; 9)(q34;q34.3) translocation together with a marker chromosome (Smith et al., 1989). Although no karyotype analysis was available for the other case, a translocation similar to t(7;9)(q34;q34.3), in which *TAN-1* DNA was joined to DNA of *TCRβ*, must have occurred in this tumor.

To investigate the exact position of translocation breakpoints in these two new cases of T-ALL containing *TAN-1* rearrangements, phage libraries constructed with size-selected DNA fragments were screened with the PS 1.3 probe. Sequence analyses of DNA inserts from hybridizing phage clones showed that the breakpoint in *TAN-1* had occurred only 5 bp apart in the two cases and less than 100 bp from the breakpoint in the original t(7; 9)(q34;q34.3) translocation (Figure 7). The *TAN-1* and

TCRβ J sequences are separated by 12 or 13 bp in the two cases by guanine-rich sequences resembling *TCRβ* diversity (D) segments, although some or all of these interposed nucleotides could represent random N sequence insertions, as are frequently seen in normal immunoglobulin and TCR gene rearrangements.

Sequence analysis of the genomic PS 1.3 probe compared with the *TAN-1* cDNA sequence showed that PS 1.3 contains one complete exon and part of a second more 3′ exon (Figure 7). The complete exon of PS 1.3 encodes the last two and a half EGF-like repeats and 2 of the 3 *lin-12/ Notch* cysteine repeats. The chromosome 9 breakpoints in the three translocations studied occurred within intronic DNA of the PS 1.3 probe 350, 257, and 252 bp 5′ of this exon. Therefore, the translocation divides the *TAN-1* coding sequence roughly in half, separating the portion encod-
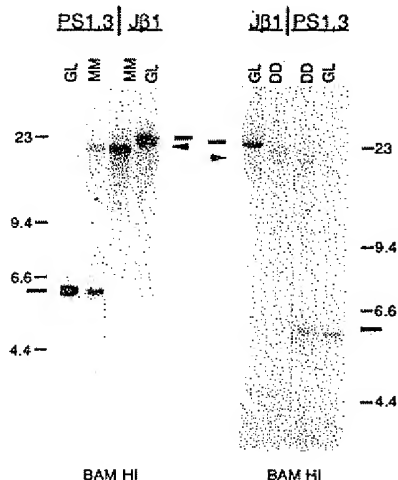
Figure 6. Southern Blot Analysis of Tumor Cell DNA Showing Comigration of Rearranged Bands Detected with the PS 1.3 (*TAN-1*) and TCR J$_b$1 Probes

DNA was extracted from pleural effusion cells of patients MM (case 3) and DD (case 2), both of whom had been diagnosed with acute T-ALL, and compared by Southern blot hybridization with human germline (sperm) DNA. Dashes indicate germline fragments; arrowheads indicate rearranged fragments. Lanes labeled PS 1.3 show Southern blot analyses of BamHI digests hybridized with the PS 1.3 probe, which contains DNA sequences adjacent to the chromosome 9 breakpoint originally cloned from case 1. After stripping the blots initially hybridized with the PS 1.3 probe, the same blots were rehybridized with a probe for the *TCRβ* J$_b$1 region.

Figure 7. Nucleotide Sequence of Translocation Breakpoints Involving *TAN-1* and *TCRβ* Loci in T-ALL Cases 2 and 3

Shown at the top is a diagram of the positions of the three translocation breakpoints in cases 1, 2, and 3 relative to the coding region of *TAN-1* (cross-hatched boxes). The diagram is oriented left to right, with respect to normal 5′ to 3′ *TAN-1* transcription. The breakpoint-proximal exon encodes two of the EGF-like cysteine repeats and one *Notch/lin-12* cysteine repeat. DNA sequences surrounding the der(9) translocation breakpoints are shown below in comparison with the germline 9 sequences from the breakpoint region. The sequence shows the template strand of *TAN-1* joined to the nontemplate strand of *TCRβ*. Joining (J) regions of the *TCRβ* gene are boxed. N region (N) and diversity region (D) sequences are presumed to lie between the *TAN-1* and J sequences.

ing most of the extracellular domain (translocated to the der(7) chromosome) from the portion encoding the transmembrane and intracellular domains (retained on the der(9) chromosome).

Analysis of *TAN-1* Transcription in Cells with Translocated and Nontranslocated *TAN-1* Genes

Figure 8A shows the results of RNAase protection studies on a variety of human tissues and cell lines. The probe used in these studies is a *TAN-1* cDNA fragment, identical to that illustrated in Figure 5A, containing parts of two TAN-1 exons that lie on either side of the chromosome 9 breakpoints. The probe should therefore distinguish between translocated and normal untranslocated *TAN-1* transcripts on the basis of having the two exons protected by RNA as a single fragment (untranslocated transcript) or as two separate fragments (translocated transcript). As expected, FL18, Jurkat, and normal peripheral blood lymphocytes all show one major band corresponding to the normal *TAN-1* transcript. In the SUP-T1 cell line, the assay shows no protected band in the position of the normal transcript—a finding consistent with the observation that SUP-T1 had duplicated the der(9) chromosome containing the 3′ end of *TAN-1* and lost the normal chromosome 9 present in the tumor from which the cell line had been derived (Reynolds et al., 1987). Instead, a major protected
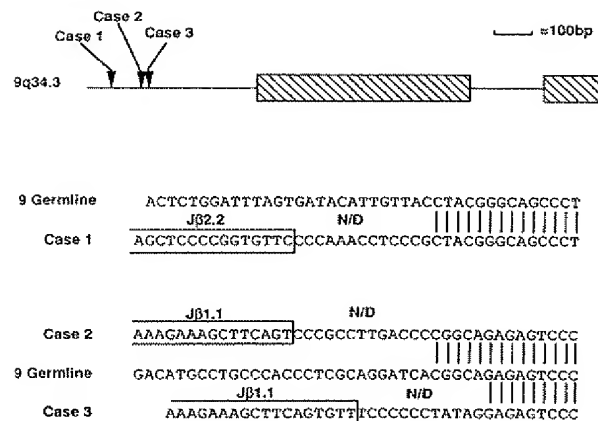
band of about 280 nucleotides was seen, corresponding to the size of the portion of the 3′ exon contained within the probe. This result suggests that SUP-T1 expresses a relatively abundant transcript from the der(9) chromosome, that this transcript is transcribed in the normal direction of *TAN-1* transcription, and that it may initiate at or 5′ to the beginning of this exon. A second major protected band of 110 nucleotides was also present (but not shown in Figure 8A), the size of which corresponds to the protected portion of the 5′ exon of the probe. Therefore, transcription of *TAN-1* sequences on the der(7) chromosome in SUP-T1 occurs in the normal *TAN-1* orientation and includes *TAN-1* coding sequences near the breakpoint. Additionally, there are a number of other bands in the analysis of SUP-T1 RNA that lie in positions between about 280–175 nucleotides in size and could represent transcripts either initiating within the 3′ exon or splicing into it. In any event, the presence of multiple bands suggests marked heterogeneity of *TAN-1* transcripts in the SUP-T1 cell line.

Northern blot analysis for RNA from the SUP-T1 cell line (Figure 8B), produced results compatible with the RNAase protection studies, indicating that both the 5′ half and the 3′ half of *TAN-1* are transcribed, although at very different levels. Lower molecular weight transcripts detected with the PS 1.3 probe confirm that several RNAs are produced from DNA near the der(9) breakpoint.

RNA from primary tumor cells in pleural effusions from two cases were studied by RNAase protection, as illustrated in Figure 8A. Previous Southern blot analysis of both cases indicated that the cells within the pleural effusions
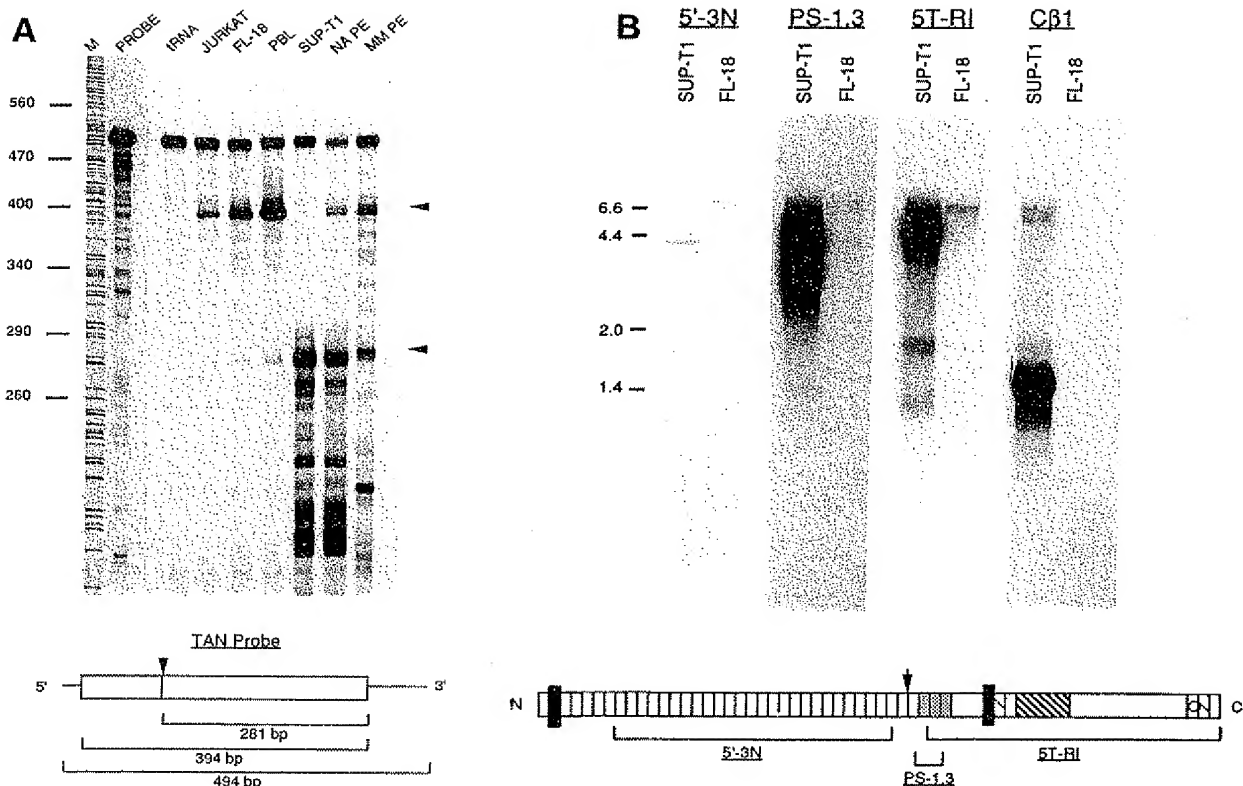
Cell
656

Figure 8. Analyses of TAN-1 Transcripts in Tumor Cells Carrying the Translocation and in a Tumor-Derived Cell Line

(A) RNAase protection analysis of TAN-1 transcripts in cells containing or lacking the t(7;9)(q34;q34.3) translocation. RNA was isolated from cells lacking the translocation (the Jurkat cell line, FL18 cell line, and normal peripheral blood lymphocytes [PBL]) and cells known to contain the translocation (pleural effusion [PE] cells from patient NA [case 1] and MM [case 3] and the SUP-T1 cell line, which was established from the PE cells of NA). The SUP-T1 cell line is known to contain two copies of the der(9) chromosome and no normal copies of chromosome 9. FL18 was established from a B cell lymphoma and contains two unrearranged copies of the TAN-1 gene. Radiolabeled antisense RNA probe was hybridized to 20 µg of total RNA in each analysis. The diagram of the probe is shown at the bottom. It contains parts of two TAN-1 exons that lie on either side of the translocation breakpoints (represented by the arrowhead) within an intervening intron. An arrowhead has been placed alongside bands corresponding to the normal TAN-1 transcripts in the Jurkat, FL18, and PBL lanes, at the position of 400-nucleotide-long products. Another arrowhead has been placed beside bands of about 280 nucleotides, corresponding to products expected from translocated TAN-1 genes.

(B) Northern blot analysis of SUP-T1 and FL18 cell line RNA with TAN-1 and TCRβ probes. The same Northern blot was hybridized sequentially with various probes, which were eluted or left to decay between hybridizations. Two micrograms of poly(A)+ RNA was run in each lane. Shown at the bottom is a diagram of the TAN-1 coding region, with various domains of homology represented as in Figure 3. The approximate location of each of the three TAN-1 probes is indicated. PS 1.3 is a genomic fragment, as described above. The arrow shows where translocation separates the 5' and 3' portions of the coding sequence.

were almost exclusively derived from the malignant clone in each case. RNA from both the SUP-T1 cell line and the pleural effusion cells from which this cell line was derived protected an RNA fragment about 280 nucleotides long, corresponding to the product expected with TAN-1 transcription from the der(9) translocated chromosome. Additionally, RNA from the pleural effusion cells, but not from the SUP-T1 cells, protected an RNA fragment of about 400 nucleotides—the size of the RNA fragment expected to be protected by normal TAN-1 transcripts. These results are in accord with the loss of the normal, untranslocated chromosome 9 observed after the pleural effusion cells were placed in culture. The complex pattern of bands between positions of about 280 and 175 nucleotides is virtually the same between the pleural effusion cells and the

SUP-T1 cell line, even though RNA was harvested from the SUP-T1 cells after more than 1 year of continuous passage of the cells in culture.

RNA from the pleural effusion cells of the second case protected a band at about 400 nucleotides, as well as a band slightly larger than the major band protected in the first case. These two protected bands probably correspond to TAN-1 transcripts from the normal untranslocated chromosome 9 and the der(9) chromosome, respectively. Another major band also appears at about 210 nucleotides and presumably derives from transcription of der(9) chromosome sequences that are contained within the 3' exon of the TAN-1 probe. Unfortunately, insufficient numbers of tumor cells from the third case were available for RNA analysis.

Discussion

TAN-1 Is the Human Homolog of Drosophila *Notch*

Comparison of the *TAN-1* and *Notch* cDNA sequences reveals that the two genes are homologous virtually throughout their entire protein coding regions. The amino acid sequences deduced from the cDNAs are 46% identical and 62% similar with respect to conservative changes in amino acids. Local regions within the sequences show even greater similarity than this. Overall, the two genes are much closer in structure than are other genes that have been considered previously to show partial homology to *Notch*.

A striking aspect of the homology between the *TAN-1* and *Notch* genes is the conservation of a series of amino acid sequence motifs that have been inferred to represent functional protein domains having specific locations within cells expressing the *Notch* product. In fact, unlike other *Notch*-related genes, such as *lin-12* and *glp-1*, *TAN-1* shares with *Notch* all recognizable motifs present within the *Notch* sequence (i.e., all motifs common to *Notch* and any other known gene). The likely functional significance of the conserved motifs in *TAN-1* and *Notch* is underscored by the finding that these motifs are the most similar portions of the sequences. Furthermore, the distances between these motifs within the sequences are comparable between *TAN-1* and *Notch*, so that the two amino acid sequences can be easily aligned except for a few gaps near the C-termini of the cDNAs.

Among the motifs found in *TAN-1* and *Notch* are two types, the EGF-like repeat and the *Notch/lin-12* repeat, which contain highly conserved cysteines and are identifiable in all *Notch*-related genes. Each of these genes is presumed to encode integral membrane proteins, and both of these cysteine repeats are considered likely to correspond with extracellular domains of these proteins — a prediction confirmed in studies using anti-Notch antibodies on intact Drosophila cells to demonstrate that the *Notch* product is a membrane-bound protein with the EGF-like repeats located in the extracellular space (Kidd et al., 1989).

The EGF-like repeat region in *Notch* has been hypothesized to mediate extracellular interactions, possibly as a receptor for some diffusible ligand, binding site in the tissue matrix, or recognition structure on certain cells. Genetic and biochemical studies have indicated that the *Notch* protein binds in a calcium-dependent manner to the product of *Delta*, another neurogenic gene in Drosophila (Fehon et al., 1990). Like *Notch*, the *Delta* gene also encodes a transmembrane protein containing extracellular EGF-like repeats, and the data currently available on the nature of *Notch–Delta* protein interaction are consistent with binding occurring through association of some or all of the EGF-like repeats within the two proteins. Whatever functions are actually served by the EGF-like repeats of the *Notch* protein, two lines of evidence suggest that individual repeats are not functionally equivalent. First, the spacing between cysteine residues, which varies among the individual repeats, is evolutionarily conserved among *TAN-1*, *Notch*, and *Xotch*. Second, mutations in individual repeats

within the *Notch* gene have different genetic properties. For example, mutations in some repeats are dominant and apparently interfere with essential *Notch–Notch* protein interactions, while other mutations seem to inhibit interactions with other neurogenic genes in Drosophila, such as *Enhancer of split* (Kelley et al., 1987).

The second set of cysteine-containing repeats, the *Notch/lin-12* repeats, may also be involved in extracellular interactions, as hypothesized for the domain of EGF-like repeats, or may conceivably be involved in transduction of signal resulting from binding of the EGF-like repeats to some extracellular molecule.

The third motif common to *TAN-1* and *Notch* contains hydrophobic amino acids in both proteins and probably spans the plasma membrane. This region is followed by the fourth motif, the so-called *cdc10/SWI6* repeat, which presumably lies intracellularly with respect to the transmembrane region of the proteins and is the only intracellular motif shared by all members of the *Notch/lin-12* group. The repeat also occurs in a number of proteins other than members of the *Notch/lin-12* group, including several times in host range proteins of vaccinia and cowpox viruses (Gillard et al., 1986; Spehner et al., 1988), 22 times at the N-terminus of the human erythrocyte protein ankyrin (Lux et al., 1990), and seven times in the deduced product of another human gene, termed *BCL-3*, which was recently identified at the breakpoint of a chromosomal translocation found in several cases of chronic lymphocytic leukemia (Ohno et al., 1990).

Since *cdc10/SWI6* repeats are found in genes that regulate events in the cell cycle or affect decisions in cell fates, the inference has been made that protein domains containing these repeats have some role in the control of cell growth and differentiation. Indeed, the suggestion has been made that such domains might bind directly to DNA, based on the presence of the repeat in the yeast gene *SWI4*, the product of which forms part of a nuclear protein complex that binds to DNA and activates transcription (Andrews and Herskowitz, 1989). However, given the putative transmembrane character of the *TAN-1* and *Notch* products, DNA binding seems an unlikely function for the *cdc/SWI6* repeats in these proteins. It seems more reasonable that domains containing this repeat may be involved generally in protein–protein interactions. For example, *cdc10/SWI6* repeats at the N-terminus of ankyrin form a domain that interacts with integral membrane proteins and tubulin (Lux et al., 1990). In view of this observation, it seems possible that the *cdc10/SWI6* repeats in the *Notch/lin-12* family interact with other intracellular proteins that are part of a signal transduction cascade. *Enhancer of split*, which has been shown genetically to interact with and function downstream of *Notch* in the control of epidermal differentiation, is a candidate signal transducing gene in the *Notch* pathway. Sequencing of this gene shows that it is homologous to the β subunit of mammalian G proteins, a result consistent with the role of this gene in signal transduction.

Two final motifs appear in the *TAN-1* and *Notch* cDNAs between the *cdc10/SWI6* repeats and the C-termini of the coding sequences. These are the opa repeat and the PEST sequence. Opa repeats were initially identified in

several Drosophila homeoproteins and have since been found in various non-homeoproteins. Because the sequence is unusually hydrophilic, it has been speculated that the presence of the sequence increases susceptibility to proteolysis and therefore serves to destabilize the protein (Wharton et al., 1985).

PEST sequences have also been considered to affect protein stability, since they have been found in a wide variety of rapidly degraded eukaryotic proteins (Rogers et al., 1986). The actual roles both of the PEST sequences and the opa repeats in *TAN-1* and *Notch* are unclear, since immunoprecipitation of pulse-labeled *Notch* protein suggests that it is stable during embryogenesis over at least several cell divisions.

Structure and Expression Data Imply Multiple Functions for *Notch* and *TAN-1*

Genes of the *Notch/lin-12* group have received attention primarily for their functions in cell lineage determination during embryonic development, as revealed by genetic studies. *Notch* is one of a class of seven neurogenic genes in Drosophila, all of which are required for correct segregation of epidermal from neuronal cell precursors during embryogenesis. Homozygous null mutants in any of the neurogenic loci are lethal during embryogenesis, since the neuronal precursors proliferate at the expense of epidermal precursors (Lehmann et al., 1983). The two C. elegans genes *lin-12* and *glp-1* are involved in the development of vulval progenitor cells and differentiation of germline cells, respectively, within the nematode (Yochem and Greenwald, 1989). It was therefore unexpected that *TAN-1*, a gene associated with a chromosomal translocation in T lineage neoplasms, should show homology to any of these genes and such extensive homology to *Notch*.

However, several considerations, mostly based on results from analyses of *Notch*, suggest that function of these related genes may not be limited to embryogenesis. For example, some mutations in *Notch* have no apparent effect during embryo development but are lethal at the larval and pupal stages (Kelley et al., 1987). Furthermore, *Notch* is expressed in many larval, pupal, and adult tissues, implying that *Notch* functions later than in embryogenesis and in fully differentiated cells (Kidd et al., 1989). In this respect, expression of *TAN-1* is similar to that of *Notch*. *TAN-1* RNA is found in many fetal and adult tissues, although the highest levels of RNA seem to occur in lymphoid tissues, central nervous system, and lung.

A reasonable explanation to reconcile these findings is that *Notch*, and possibly *TAN-1*, as well, act in diverse ways at different times during development and in different tissues. Apparently, *TAN-1* has over the course of evolution acquired some additional function in lymphocytes, a cell type that does not exist in insects or nematodes. Such a connection between gene expression in lymphocytes and other kinds of tissues has numerous precedents, particularly with respect to the central nervous system (Goetzl et al., 1990; Chun et al., 1991). For example, a specific link has recently been made between a gene mapping close to a chromosomal translocation in T-ALL and neural dif-

ferentiation. This gene, which encodes a protein termed rhombatin, lies near a translocation breakpoint involving the TCRδ gene and has been shown to be selectively expressed in the rhombomeres of the developing mouse hindbrain (Greenberg et al., 1990). All of these observations point to the general principle that the developing lymphoid and nervous systems often utilize the same genes. The finding of abundant *TAN-1* RNA in lymphoid and neural tissues may be another manifestation of this principle.

Alteration of *TAN-1* Probably Contributes to the Malignant Phenotype

Detection of recurrent breakage of *TAN-1* by chromosomal translocations in a single type of cancer seems more than coincidental and provides good circumstantial evidence for the role of this gene in malignant transformation or tumor progression. Retention of the translocation in tumor cell lines (including a cell line from case MM in which the karyotype of the cell line contained only one other recognizable chromosomal abnormality; see Experimental Procedures) further supports this interpretation.

The mechanism by which recombination within the *TAN-1* gene exerts its presumed effect in ALL is at present not clear. One possibility is that *TAN-1* acts as a tumor suppressor gene, as suggested by the loss of the normal chromosome 9 from the SUP-1 cell line. This hypothesis would imply that the second *TAN-1* allele, although present, is inactivated in some more subtle way in other cell lines and tumors carrying the t(7;9)(q34;q34.3) translocation, including the tumor from which the SUP-1 line was derived. However, this hypothesis does not explain the conservation of breakpoint positions within TAN-1. It could be that the close proximity of breakpoints at this site is due entirely or in part to some purely structural feature of DNA that predisposes this region of chromosome 9 to translocation in developing lymphocytes. The feature most frequently speculated about in this regard is the existence of ectopic heptamer-like sequences that are recognized by lymphocyte recombinase to catalyze recombination between DNA adjacent to these sequences and rearranging segments of antigen receptor genes (Tycko and Sklar, 1990). The presence of TCRβ J region segments together with possible interposed D and N region nucleotides at the junction of chromosome 7 and 9 DNA lends a degree of superficial support for this notion. On the other hand, only in case 1 does the breakpoint in chromosome 9 DNA occur near a sequence resembling the canonical recombinase heptamer CACAATG (Reynolds et al., 1987).

Perhaps a more plausible explanation for tight clustering of breakpoints in *TAN-1* is that breakage at the observed site results in some crucial alteration in the *TAN-1* product. Consistent with this view is the finding of multiple major transcripts corresponding to truncated versions of the *TAN-1* gene in tissues from at least two cases of T-ALL carrying the t(7;9)(q34;q34.3) translocation. Although there is as yet no direct evidence that an altered *TAN-1* protein is produced from the translocated gene, the position of the breakpoint in all three cases of T-ALL studied suggests

that the effect of the translocation could be the equivalent of removal of the extracellular domain of the normal *TAN-1* protein. If the extracellular domain of the *TAN-1* protein mediates cell–cell interactions, as has been hypothesized for the extracellular domain of *Notch*, this interpretation would coincide with the growing concept that loss of cell adhesion receptors plays some part in malignant behavior of many different tumors. For example, transformation of various types of cultured cells by *ras* oncogenes leads to a decreased expression of several integrins (Plantefaber and Hynes, 1989), while overexpression of integrins by transfected integrin genes in Chinese hamster ovary cells suppresses the transformed phenotype in these cells (Giancotti and Ruoslahti, 1990). Also, the gene deleted in the majority of human colorectal cancers has strong homology to the N-CAM family of cell adhesion molecules (Fearon et al., 1990). Furthermore, the expression of the integrin LFA-1 is consistently low in a variety of B cell tumors and is reduced following transformation by *C-MYC* transfection of B cells immortalized with Epstein–Barr virus (Inghirami et al., 1990).

An alternative mechanism by which removal of the extracellular region of the protein may activate the oncogenic potential of *TAN-1* is to uncouple binding of some ligand by the extracellular region from some growth-controlling signal transduced by the intracellular portion of the protein. This process would be akin to that proposed for the mechanism of the *ERB-B* oncogene product, based on its homology to the intracellular part of the EGF receptor (Downward et al., 1984). In theory, the signal transduced by the intracellular portion of the *TAN-1* protein could be either inhibitory or stimulatory to growth, and loss of the extracellular region in *TAN-1* could either block transmission of an inhibitory signal or cause constitutive stimulatory signaling for cell growth.

The portion of *TAN-1* encompassing the *cdc10/SWI6* repeats seems particularly likely to be involved in signal transduction. This repeat, contained within the most highly conserved domain between *TAN-1* and *Notch*, has been implicated in protein–protein interactions in the cytoplasm (as in ankyrin and the *Notch/lin-12* group of genes) and in protein–protein or protein–DNA interactions in the nucleus' (as in SWI4 and SWI6). A similar domain composed of seven tandem *cdc10/SWI6* repeats was recently also found in the *BCL-3* gene, which has been identified at the breakpoint of a chromosomal translocation involving the immunoglobulin heavy chain gene in several cases of the B cell neoplasm chronic lymphocytic leukemia (CLL) (Ohno et al., 1990). The *BCL-3* gene, which shows increased expression in CLL cells containing the translocation and in mitogenically stimulated normal blood mononuclear cells, differs from *TAN-1* in that it contains no apparent signal sequence or transmembrane domain, and the *cdc10/SWI6* repeats have greater homology to the repeats in yeast genes than to the repeats in either *TAN-1* or *Notch*. Nevertheless, further research may yet show that *TAN-1* and *BCL-3* constitute the first members of a class of genes containing *cdc10/SWI6* repeats and associated with the control of lymphocyte proliferation and chromosomal translocations in lymphocytic neoplasms.

Experimental Procedures

Cell Lines and Tumor Specimens
The SUP-T1 cell line was established from an acute T lymphoblastic leukemia, as described previously (Reynolds et al., 1987; Smith et al., 1988, 1989), and was shown by Southern analysis to contain the same t(7;9)(q34;q34.3) translocation as the patient's tumor cells. The full karyotype of this cell line is 46,XY,inv(2)(q11q21),inv(2)(q11q21), del(4)(q31q35),del(6)(q23q27),t(7;9)(q34;q34.3),+der(9)t(7;9)(q34;q34.3), −9,inv(14)(q11.2q32) (Reynolds et al., 1987). FL18 was established from a follicular B cell lymphoma containing a t(14;18)(q32;q21) translocation, as described elsewhere (Doi et al., 1987). The Jurkat cell line was derived from an acute T cell lymphoblastic leukemia (Schwenk and Schneider, 1975). Primary tumor cells from cases 1, 2, and 3 (patients NA, DD, and MM, respectively) were obtained from either peripheral blood (NA) or pleural effusions (DD, MM) that were shown by Southern analysis to contain predominantly the tumor clone. Cells from the pleural effusion of MM were not karyotyped, but a cell line established from these cells showed the karyotype 46,XY,t(7;9)(q34; q34.3),+mar (Smith et al., 1989; the cell line derived from MM could not be revived after cryopreservation and was not available for our studies). Bone marrow from a subsequent relapse in this case showed a karyotype of 46,XY,t(7;9)(q34;q34.3),−9,11q+,12p+,+mar. Cells from DD were not karyotyped.

Nucleic Acid Isolation and Hybridization Analysis
High molecular DNA was isolated from tumor cells and cell lines by standard procedures (Cleary and Sklar, 1984). RNA from human and mouse organs was prepared, and poly(A)$^+$ RNA was selected according to the methods described in Davis et al. (1986). DNA and RNA blotting and hybridization were performed as described in Reynolds et al. (1987). DNA probes for hybridization were labeled with [α-^{32}P]dCTP using the method of random hexanucleotide priming (Feinberg and Vogelstein, 1983).

cDNA and Genomic Cloning
cDNA was prepared from the Jurkat cell line using 3 μg of poly(A)$^+$ RNA and cloned in λgt10 by methods described earlier (Cleary et al., 1986). cDNA from cultured human umbilical vein endothelial cells was synthesized and cloned as described in Ginsburg et al. (1985). cDNA was synthesized from the FL18 cell line by priming reverse transcription of poly(A)$^+$ RNA with various *TAN-1*–specific antisense oligonucleotides. Blunt-ended, double-stranded cDNA was prepared by the methods of Gubler and Hoffman (1983). The cDNA was kinased, ligated to phosphatased EcoRI linkers (Amersham Corp.; Arlington Heights, IL), then kinased again and ligated into λgt10 genomes. Recombinant phage genomes were packaged and phage libraries plated according to standard procedures (Maniatis et al., 1982), followed by screening of the resulting plaques with DNA probes using conditions identical to those for Southern hybridization. The Jurkat cDNA library was screened with the genomic PS 1.3 probe, while the FL18 and endothelial cell libraries were screened with purified *TAN-1* cDNA fragments.

Cloning of DNA containing the t(7;9)(q34;q34.3) breakpoint from the SUP-T1 cell line and the germline sequences of the chromosome 9q34.3 locus from human sperm has been described (Reynolds et al., 1987). To clone the breakpoints for cases 2 and 3, genomic DNA was double digested with BamHI and BglII restriction enzymes, size selected by agarose gel electrophoresis, and ligated into λ dash (Stratagene; La Jolla, CA). Libraries were packaged and plated as above, and screened with the PS 1.3 probe.

Subcloning of DNA Fragments and Nucleotide Sequence Analysis
Purified phage DNA from either cDNA or genomic phage libraries were restriction mapped, and appropriate fragments were subcloned into the Bluescript plasmid vector (Stratagene), or into single-stranded phage vectors M13mp18 or M13mp19 (Norrander et al., 1983) for sequence analysis. Sequence analysis was carried out using the method of Sanger et al. (1977) with a modified T7 polymerase (United States Biochemical Corp., Cleveland, OH). Sequencing reactions were primed with either the M13 universal primer or custom-made oligonucleotide primers. Sequence for the 8.3 kb composite *TAN-1* cDNA was determined on both DNA strands.

RNAase Protection Analysis

These experiments were performed by modification of the procedure described in Ausubel et al. (1987). In brief, antisense RNA probes were transcribed from Bluescript plasmids containing human or mouse *TAN-1* sequences flanked by the T3 and T7 RNA polymerase promoters. RNA was transcribed using the Stratagene RNA transcription kit and labeled with $[\alpha\text{-}^{32}P]rUTP$. Ten milligrams of sample RNA together with 5×10^5 cpm probe RNA in hybridization buffer (80% formamide, 40 mM PIPES [pH 6.7], 0.4 M NaCl, 1 mM EDTA) were denatured at 80°C for 5 min, then hybridized overnight at 50°C. Following quenching on ice, the 40 μl hybridization reactions were added to 0.3 ml of RNAase solution (20 μg/ml RNAase A and 0.7 μg/ml RNAase T1 in 10 mM Tris [pH 7.5], 5 mM EDTA, 0.3 M NaCl) and digested for 15 min at room temperature. RNAases were inactivated with proteinase K and SDS, then reactions were extracted with phenol–chloroform, precipitated in 70% ethanol, and analyzed in a 6% denaturing polyacrylamide gel.

Isolation of Mouse *TAN-1* cDNA Sequences with PCR

To isolate a cDNA probe for the mouse *TAN-1* gene, 5 μg of total RNA from either mouse thymus or spleen was used to synthesize single-stranded cDNA by priming reverse transcription with a specific antisense oligonucleotide from the *cdc10/SWI6* region of *TAN-1* (5′-GGCAGGCGGTCCATATGATCCGTGAT-3′). Ten percent of this cDNA was amplified by PCR in a standard reaction mixture provided with the Perkin Elmer Cetus PCR kit (Perkin Elmer Cetus; Norwalk, CT), to which was added 20 pmol each of the above oligomer and a sense-strand oligomer (5′-GATGCCAACATCCAGGACAACATGGG-3′) corresponding to a sequence about 450 bp 5′ of the sequence complementary to the first oligomer. PCR reaction conditions were: 1 min denaturing at 94°C, 1 min annealing at 50°C, and 2 min elongation at 70°C for 25 cycles. A single band of the predicted size was detected by ethidium bromide staining of an agarose gel following electrophoresis of the reaction products. The band was eluted from the gel, kinased, and cloned into the Bluescript plasmid vector. The fragment detected bands corresponding to the *TAN-1* gene on Southern analysis of both mouse and human DNA, but it protected bands on RNAase protection analysis only of mouse RNA.

Computer Analysis

The University of Wisconsin Genetics Computer Group software package was used on a Digital Equipment Corp. VAX 8550 computer. The initial data base search was performed with WORDSEARCH, and sequence alignment and homology were determined using the GAP (Devereux et al., 1984) and LOCAL (Smith et al., 1985) algorithms.

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Rel/NF-κB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors

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Jagged1 belongs to the DSL family of ligands for Notch receptors that control the proliferation and differentiation of various cell lineages. However, little is known about the transcription factors that regulate its expression. Here, we show that Jagged1 is a Rel/NF-κB-responsive gene. Both c-Rel and RelA induced *jagged1* gene expression, whereas a mutant defective for transactivation did not. Importantly, *jagged1* transcripts were also upregulated by endogenous NF-κB activation and this effect was inhibited by a dominant mutant of IκBα, a physiological inhibitor of NF-κB. Cell surface expression of Jagged1 in c-Rel-expressing cell monolayers led to a functional interaction with lymphocytes expressing the Notch1/TAN-1 receptor. This correlated with the initiation of signaling downstream of Notch, as evidenced by increased levels of *HES-1* transcripts in co-cultivated T cells and of *CD23* transcripts in co-cultivated B cells. Consistent with its Rel/NF-κB-dependent induction, Jagged1 was found to be highly expressed in splenic B cells where c-Rel is expressed constitutively. These results demonstrate that c-Rel can trigger the Notch signaling pathway in neighboring cells by inducing *jagged1* gene expression, and suggest a role for Jagged1 in B-cell activation, differentiation or function. These findings also highlight the potential for an interplay between the Notch and NF-κB signaling pathways in the immune system.
Keywords: Jagged1/NF-κB/Notch/Rel/TAN-1

Introduction

The Rel/NF-κB transcription factors play fundamental roles in the immune system. These structurally related proteins share common pathways of activation that involve their release from inhibitory IκB factors in response to stimuli (reviewed in Whiteside and Israel,

1997). The degradation of IκB results in the nuclear translocation of active Rel dimers, their binding to κB DNA sites and the activation of cellular gene expression (reviewed in Baldwin, 1996). NF-κB regulates the expression of various cytokines, chemokines, immunoreceptors, growth factors, acute phase proteins and adhesion molecules (reviewed in Kopp and Ghosh, 1995; May and Ghosh, 1997). It is therefore not surprising to find its implication in the immune, inflammatory and acute phase responses (reviewed in Wulczyn et al., 1996; Barnes and Karin, 1997).

The Notch gene family encodes transmembrane receptors that control cell proliferation and differentiation in response to extracellular ligands expressed on neighboring cells (reviewed in Robey, 1997; Weinmaster, 1997). Among the four mammalian Notch receptors identified to date, three of them are expressed in hematopoietic cells. Human Notch1/TAN-1 and its murine counterpart are found in spleen, CD34+ hematopoietic stems cells and early progenitor cells, thymic T cells, peripheral blood lymphocytes and a B-lymphoma cell line (Ellisen et al., 1991; Milner et al., 1994; Hasserjian et al., 1996). Similarly, Notch2 is highly expressed in spleen, and both Notch2 and Notch3 are found in hematopoietic and myeloid progenitor cell lines (Weinmaster et al., 1992; Milner et al., 1996; Bigas et al., 1998). Notch1 was isolated originally as a translocation in a human acute T-cell lymphoblastic leukemia/lymphoma, and its constitutively active form produces T-cell neoplasms in mice (Ellisen et al., 1991; Pear et al., 1996). Two other Notch genes also map to chromosomal locations associated with human immune system malignancies (Larsson et al., 1994; reviewed in Gridley, 1997).

Jagged1, Jagged2, Delta1 and Delta2 belong to the DSL (Delta/Serrate/LAG-2) family of ligands for mammalian Notch receptors (reviewed in Weinmaster, 1997). All are transmembrane proteins with an extracellular domain important for receptor binding. Ligand-mediated activation of Notch induces the proteolytic release of the intracellular domain of Notch, activation of cellular gene expression and suppression of differentiation (Schroeter et al., 1998; reviewed in Robey, 1997; Weinmaster, 1997; Bray, 1998; Chan and Jan, 1998). Studies point to the CSL [CBF1/Su(H)/Lag-1/RBP-Jκ/KBF2] family of DNA-binding proteins as downstream effectors in this pathway (Brou et al., 1994; Dou et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Jarriault et al., 1995; Tamura et al., 1995; Hsieh et al., 1996). In the nucleus, CSL factors serve as docking proteins that direct the intracellular domain of activated Notch to the promoter of target genes (Jarriault et al., 1995; Hsieh et al., 1996; Kopan et al., 1996; Schroeter et al., 1998; reviewed in Bray, 1998; Chan and Jan, 1998). The mammalian basic helix–loop–helix (bHLH) transcription factor Hairy Enhancer of Split

(HES-1) and the B-cell activation marker CD23 are two cellular target genes directly regulated by this pathway (Wang *et al.*, 1987, 1991; Cordier *et al.*, 1990; Sasai *et al.*, 1992; Ling *et al.*, 1994; Bailey and Posakony, 1995; Jarriault *et al.*, 1995, 1998; Lecourtois and Schweisguth, 1995; de la Pompa *et al.*, 1997). Little is known of the factors that trigger ligand-mediated signaling upstream of Notch.

In this report, we identified *jagged1* as a novel Rel/NF-κB-responsive gene. We show that *jagged1* gene expression can be induced specifically by transcriptionally competent NF-κB subunits and that it is suppressed by a super-IκBα inhibitor (IκBαΔN). Our finding that NF-κB-mediated induction of Jagged1 can initiate signaling downstream of Notch correlates with the expression of Jagged1 in peripheral lymphoid tissues. These results suggest a direct interplay of the Notch and NF-κB signaling pathways in the immune system, both of which have been implicated in lymphoid cell proliferation and function.

Results

Ectopic Rel protein expression induces jagged1 transcripts

The CCR43 cell line conditionally expresses c-Rel under the control of a tetracycline-regulated transactivator (tTA), resulting in inducible transactivation of Rel target genes such as *IκBα* (Bash *et al.*, 1997). An mRNA differential display analysis identified a 106 bp cDNA fragment that was induced specifically in CCR43 cells expressing c-Rel. When used as a probe in Northern blots, this fragment hybridized to a 5.8 kb transcript induced in response to c-Rel expression in CCR43 cells (data not shown). A complete cDNA was isolated from a human HeLa cell library (clone 14-6; 5457 bp). This clone showed 90% homology to rat *jagged1* and was virtually identical to two human *jagged1* cDNA clones that were isolated recently (Lindsell *et al.*, 1995; Oda *et al.*, 1997; Li *et al.*, 1998). Clone 14-6 contained a 3657 bp open reading frame encoding a 1218 amino acid human Jagged1 protein, along with 263 bp of 5′-untranslated sequence and 1537 bp of 3′-untranslated sequence (DDBJ/EMBL/GenBank accession No. AF028593). Two amino acid differences were found between the Jagged1 protein sequence reported by Oda *et al.* (1997) and the sequence described by us and by Li *et al.* (1998).

The differential expression of *jagged1* was characterized further by Northern blot analysis of CCR43 cells during a time course of c-Rel induction, using the *jagged1* cDNA as a probe (Figure 1A). *jagged1* transcripts were induced with kinetics that paralleled those for c-*rel* and for the Rel/NF-κB target gene encoding IκBα (lanes 10–18). No induction was seen in the parental HtTA-1 cells that expressed the tTA activator alone (lanes 1–9), indicating that *jagged1* is under c-Rel control. The transactivation of *jagged1* was specific, as endogenous transcripts for the ligand Delta1 and for the Notch1/TAN-1 receptor were unaffected (Figure 1B, lanes 1 and 2). Importantly, a cell line expressing a mutant c-Rel protein deleted of its transactivation domain (denoted CCR-H5) failed to upregulate *jagged1* transcripts (Figure 1B, lanes 3 and 4). Similar results were observed with cells expressing

p50/NF-κB1, a subunit of NF-κB that fails to activate gene expression from most promoters containing a κB DNA site (data not shown). In contrast, *jagged1* transcripts were induced in HtTA-RelA cells that conditionally expressed the RelA transactivating subunit of NF-κB, although some leaky expression of *jagged1* was observed in these cells in the presence of tetracycline (Figure 1C, lanes 1 and 2). Together, these results indicated that the transcriptional activity of c-Rel and RelA was necessary to activate *jagged1* gene expression.

jagged1 gene expression is dependent upon endogenous NF-κB activation

To investigate further the functional linkage between *jagged1* and Rel/NF-κB factors, we monitored the effect of endogenous NF-κB activation on *jagged1* gene expression. Human Jurkat T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin to activate endogenous NF-κB factors. This treatment led to a 4-fold increase in *jagged1* mRNA levels in comparison with unstimulated cells (Figure 1D, compare lanes 1 and 2). Similar results were obtained in human CEM T cells (data not shown). Importantly, the constitutive repression of endogenous NF-κB by a dominant mutant of IκBα in Jurkat T cells interfered strongly with the induction of *jagged1* in response to PMA plus ionomycin (Figure 1D, lanes 3 and 4; Boothby *et al.*, 1997; Chu *et al.*, 1997). Rehybridization to an interleukin-2 receptor alpha (IL-2Rα) probe confirmed the activation of NF-κB in Jurkat T cells treated with PMA plus ionomycin, and the reduction in NF-κB activation in Jurkat-IκBαΔN cells (Figure 1D, lanes 2 and 4). These results agreed with our findings in the CCR43 and HtTA-RelA cell lines and indicated that the expression of *jagged1* was dependent on Rel/NF-κB activity.

Cell surface expression of Jagged1 promotes cell–cell interactions

The expression of c-Rel led to increased levels of Jagged1 protein in CCR43 cells. Immunoblot analyses using a monoclonal antibody specific for the unique intracellular domain of human Jagged1 (Ab #TS1) showed the accumulation of Jagged1 in response to c-Rel induction (Figure 2A, lanes 1–5). Immunofluorescence analysis of live non-permeabilized cells using an antibody directed against the extracellular domain of Jagged1 verified the localization of the protein at the surface of CCR43 cells expressing c-Rel (Figure 2B, panel c). In contrast, only background staining was detected in CCR43 cells cultured in the presence of tetracycline to prevent c-Rel expression (panel b). Taken together, these results indicated the accumulation of the Jagged1 protein at the cell surface in response to c-Rel expression.

The Notch signaling pathway is triggered by the interaction of cells expressing Notch receptors with cells that express Notch ligands at their surface. We tested whether the Rel-dependent induction of Jagged1 in CCR43 cells could promote a functional interaction with cells expressing endogenous Notch1/TAN-1 receptors in co-cultivation assays. As shown in Figure 3A, non-adherent human Jurkat T cells that expressed a wild-type *TAN-1* gene aggregated onto a monolayer of CCR43 cells induced to express c-Rel (panel b). This interaction was observed
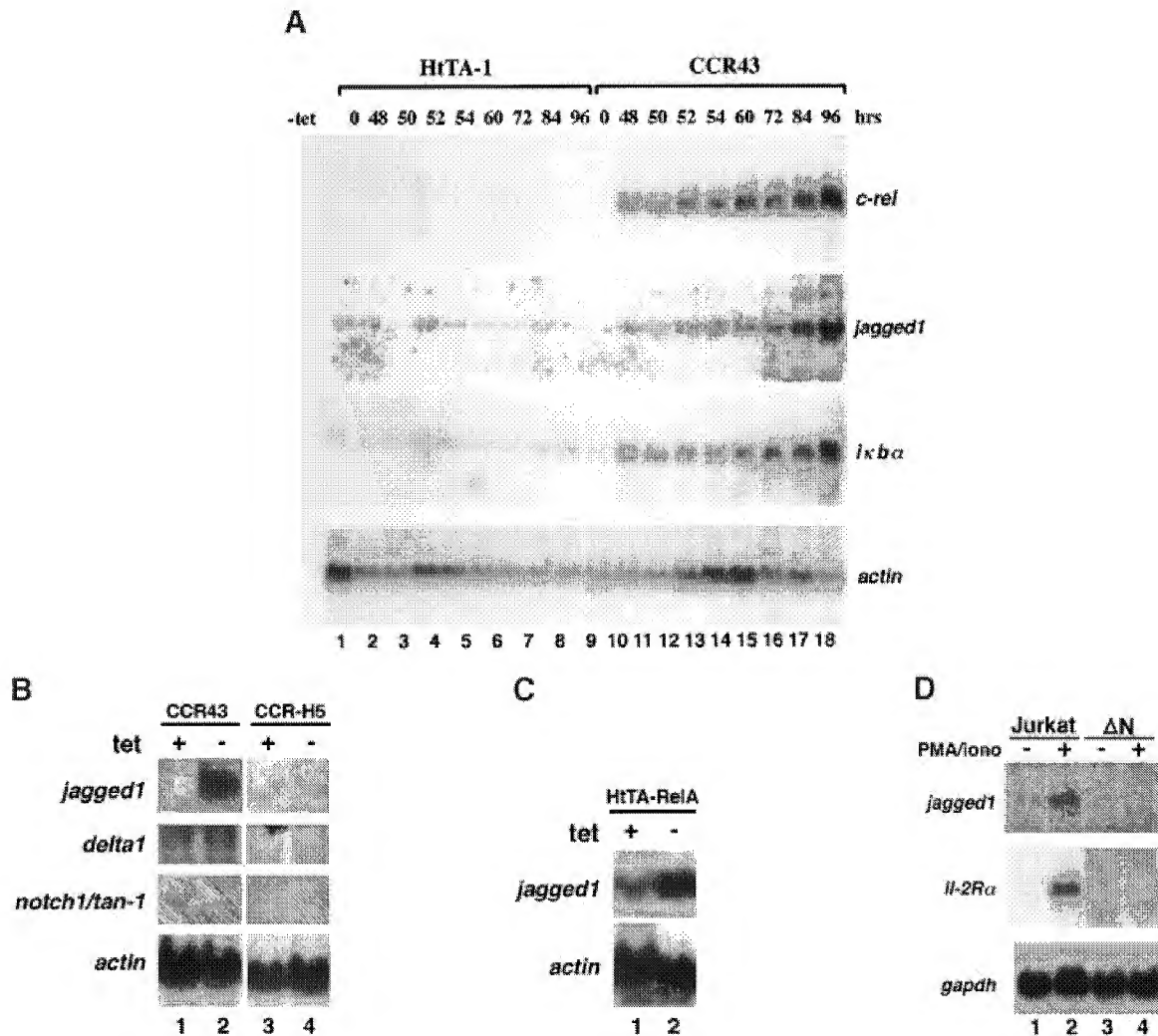
Fig. 1. Rel/NF-κB-dependent induction of *jagged1* expression. (**A**) Northern blot analysis of *jagged1* expression in CCR43 cells, during a time course of c-Rel induction. RNA from control HtTA-1 cells (lanes 1–9) and CCR43 cells (lanes 10–18) maintained in the presence (lanes 1 and 10) or absence (lanes 2–9 and 11–18) of tetracycline, was analyzed using human *jagged1* cDNA clone λ2-3 as a probe. The blot was rehybridized successively to control c-*rel*, *IκBα* and actin gene probes. (**B**) Comparative Northern blot analysis of *jagged1*, *delta1* and *Notch1/TAN-1* transcripts in cells expressing wild-type c-Rel or a mutant c-Rel protein deleted of its transactivation domain. CCR43 (lanes 1 and 2) and CCR-H5 cells (lanes 3 and 4) were cultured in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of tetracycline. Expression was analyzed with probes derived from the human *jagged1* cDNA clone λ2-3, a rat *delta1* cDNA and a human *Notch1/TAN-1* cDNA fragment. Rehybridization to actin was used as a control. (**C**) Northern blot analysis of *jagged1* expression in HtTA-RelA cells expressing the RelA protein. HtTA-RelA cells were cultured in the presence (lane 1) or absence of tetracycline to induce RelA expression (lane 2). Expression was analyzed using the human *jagged1* cDNA clone λ2-3 as a probe. Rehybridization to actin was used as a control. (**D**) Induction of *jagged1* gene expression in response to endogenous NF-κB activation in T cells. Jurkat T cells and a Jurkat T-cell clone that expressed a dominant IκBαΔN inhibitor of Rel/NF-κB factors were left untreated (lanes 1 and 3), or were stimulated with PMA plus ionomycin for 2 h to activate endogenous NF-κB factors (lanes 2 and 4). Total cellular RNA (20 μg) was analyzed for the expression of *jagged1* transcripts in Northern blots. The blot was rehybridized successively to Il-2Rα and *gapdh* probes as controls.

within 2 h of co-cultivation. Whereas gentle washings failed to disrupt the cell aggregates that formed on c-Rel-expressing cell monolayers, few T cells remained attached to CCR43 cells that were not induced to express c-Rel (Figure 3A, compare panels a and b). No aggregation was observed when Jurkat T cells were cultivated with cell monolayers expressing a mutant c-Rel protein deleted of its transactivation domain (CCR-H5 cells) or with the parental HtTA-1 cells (panels c and d; data not shown). These results agreed with our finding that the transcriptional activity of c-Rel was necessary to induce the expression of *jagged1*.

Although the data suggested the interaction of Jagged1

and Notch proteins expressed at the surface of CCR43 and Jurkat T cells, respectively, it remained possible that cytokines or cell adhesion molecules induced by c-Rel could be responsible for the observed cell interactions. We therefore investigated whether an intact Notch receptor was required for cell aggregation, by testing the ability of CCR43 cells to promote the aggregation of human Sup-T1 T cells. Both Jurkat T cells and Sup-T1 T cells are derived from a human T-lymphoblastic leukemia. While Jurkat T cells lack any known abnormality of chromosome 9 and express an intact Notch1/TAN-1 receptor, Sup-T1 T cells contain two copies of a t(7;9) (q34;q34.3) translocation and have no normal *Notch/*
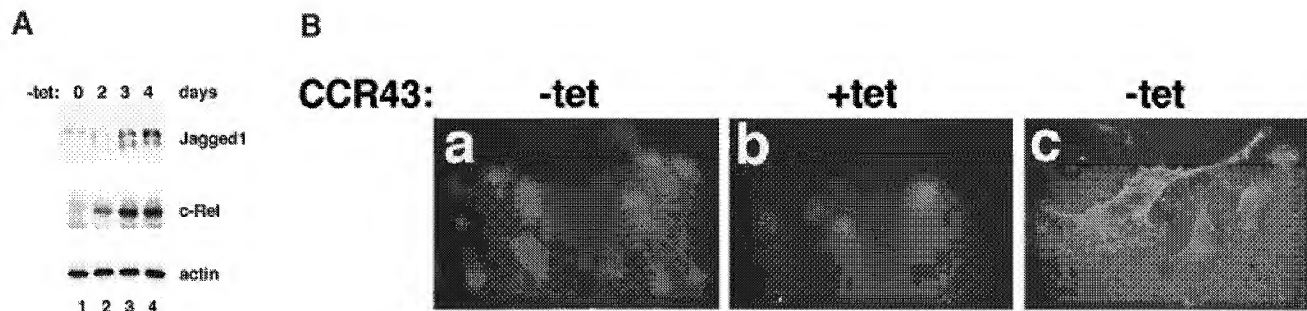
A

B

Fig. 2. Jagged1 protein expression in CCR43 cells. (**A**) Immunoblot analysis of the Jagged1 protein in CCR43 cells induced to express c-Rel. CCR43 cells were left uninduced (lane 1) or were induced to express c-Rel (lanes 2–4). Cell extracts (20 µg) were resolved by SDS–5% PAGE and analyzed by ECL immunoblotting using monoclonal anti-human Jagged1 antibody #TS1. Immunoblotting for c-Rel and actin were used as controls. (**B**) Immunofluorescence analysis of Jagged1 protein expression at the surface of CCR43 cells induced to express c-Rel. Live non-permeabilized CCR43 cells were left uninduced (panel b) or were induced to express c-Rel (panels a and c), and stained with polyclonal anti-Jagged1 antibody #PCR8 (panels b and c). CCR43 cells induced to express c-Rel were stained with a pre-immune antibody as a control (panel a).
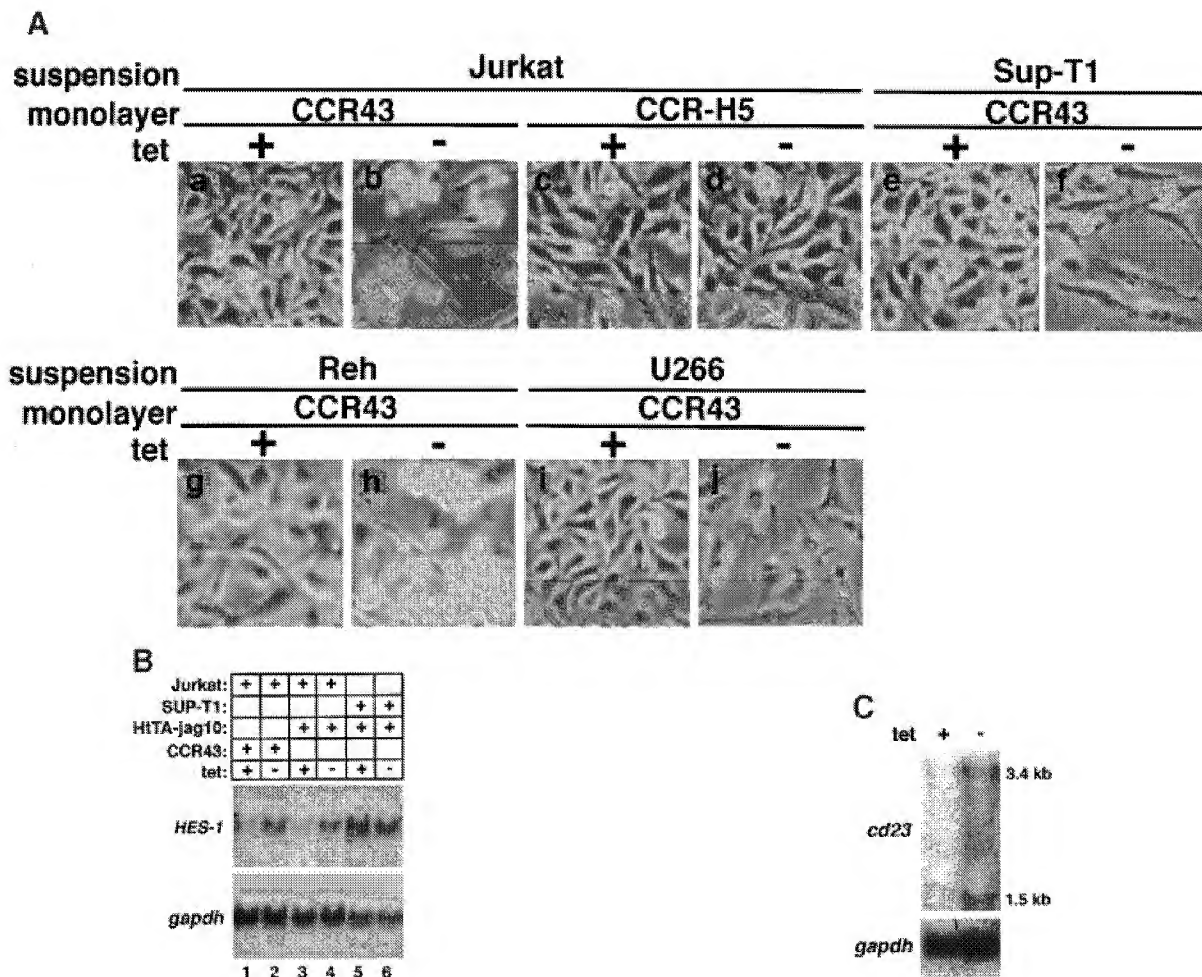
A

B

C

Fig. 3. Functional interaction between c-Rel-expressing cells and cells expressing endogenous Notch1/TAN-1 receptors. (**A**) Co-cultivation assay. Non-adherent human Jurkat T cells (a–d), Sup-T1 T cells (e and f), Reh pre-B cells (g and h) or U266 B cells (i and j) were co-cultivated onto adherent monolayers of uninduced (a, c, e, g and i) or induced (b, d, f, h and j) CCR43 cells (a, b and e–j), or CCR-H5 cells (c and d) for 48 h. After co-cultivation, the non-adherent cells were gently washed away and the cells that remained attached onto the cell monolayers were photographed. In agreement with our previous studies showing a c-Rel-mediated growth arrest in CCR43 cells, the density of the induced CCR43 cell monolayers in panels b, f, h and j is lower than that in panels a, c, d, e, g and i. (**B**) Northern blot analysis of endogenous *HES-1* gene expression in Jurkat T cells (lanes 1–4) or Sup-T1 T cells (lanes 5 and 6) co-cultivated onto cell monolayers of uninduced or induced CCR43 cells (lanes 1 and 2), and uninduced or induced HtTA-jag10 cells (lanes 3–6). The blot was rehybridized to a *gapdh* probe as a control. (**C**) Northern blot analysis of endogenous *CD23* gene expression in human Reh pre-B cells cultivated onto a CCR43 cell monolayer uninduced (lane 1) or induced to express c-Rel (lane 2). The blot was rehybridized to a *gapdh* probe as a control.

TAN-1 gene (Smith *et al.*, 1988; Aster *et al.*, 1994). The mutant TAN-1 protein in Sup-T1 T cells therefore lacks the extracellular domain necessary for ligand interaction

(Ellisen *et al.*, 1991; Aster *et al.*, 1994). Consistent with this observation, the induction of c-Rel in CCR43 cells failed to induce the aggregation of Sup-T1 T cells

(Figure 3A, panel f). This is in sharp contrast to the cell aggregation that was observed with Jurkat T cells (Figure 3A, panel b). Similarly, human Reh pre-B cells that expressed high levels of endogenous Notch1/TAN-1 transcripts aggregated onto the CCR43 cell monolayer induced to express c-Rel. In contrast, human U266 mature B cells that did not express any TAN-1 failed to do so (compare panels h and j; Guan et al., 1996; data not shown). While it is possible that other factors may also contribute to these interactions, the results indicated that an intact Notch extracellular domain was necessary for the observed cell–cell interactions and suggested that c-Rel enabled a functional interaction between Jagged1 and Notch.

c-Rel leads to activation of the Notch signaling pathway

Ligand binding to the extracellular domain of Notch initiates signaling downstream of the receptor. The genes encoding the mammalian transcription factor HES-1 and the B-cell activation marker CD23 are two direct downstream targets of this signaling pathway (Wang et al., 1987, 1991; Cordier et al., 1990; Ling et al., 1994; Bailey and Posakony, 1995; Jarriault et al., 1995, 1998; Lecourtois and Schweisguth, 1995). We examined whether the c-Rel-dependent induction of Jagged1 could trigger Notch signaling in neighboring cells by monitoring its effect on endogenous HES-1 and CD23 gene expression in co-cultivated T cells and B cells, respectively. HES-1 transcripts reproducibly were increased 2- to 3-fold in Jurkat T cells cultivated onto a monolayer of CCR43 cells expressing c-Rel in comparison with those cultivated with an uninduced CCR43 cell monolayer (Figure 3B, lanes 1 and 2). Importantly, HES-1 transcripts were also induced in Jurkat T cells cultivated onto a HtTA-1-derived cell monolayer that expressed Jagged1 alone (HtTA-Jag10 cells; compare lanes 3 and 4). Rehybridization to a control gapdh probe confirmed the specificity of this induction. As anticipated, high levels of HES-1 transcripts were observed in the control Sup-T1 T cells that express a truncated and constitutively active Notch-1/TAN-1 receptor (lanes 5 and 6). In agreement with these findings, the co-cultivation of human Reh pre-B cells with a CCR43 cell monolayer expressing c-Rel resulted in the induction of CD23 gene expression, in comparison with Reh cells cultivated onto an uninduced CCR43 cell monolayer (Figure 3C). Together, these data support a model whereby c-Rel can trigger Notch signaling in neighboring cells by inducing the expression of Jagged1.

jagged1 is highly expressed in peripheral lymphoid organs and in splenic B cells

The induction of jagged1 gene expression in response to endogenous Rel/NF-κB activation led us to examine its expression in immune tissues by Northern blot analysis. High levels of jagged1 transcripts were found in human spleen and lymph nodes, in agreement with the high levels of c-rel transcripts in these tissues (Figure 4A, lanes 1 and 2). Lower levels of jagged1 mRNAs were seen in human thymus, bone marrow and fetal liver (lanes 3, 5 and 6). No expression was detected in peripheral blood leukocytes (PBL; lane 4).

Immunohistochemistry analysis using a monoclonal antibody specific for the intracellular domain of the

Jagged1 protein was used to investigate its expression in adult mouse spleen. Strong Jagged1 staining was confined to the B-cell areas of the spleen (Figure 4B, panel a). Positive cells were located mainly in the marginal zone. A weaker signal was detected in the perifollicular zone, the periarterial sheath and the red pulp. Jagged1 was found to co-localize with the B-cell antigen CD20 (compare panels a and c). In contrast, only background staining was observed with normal rat serum (panel b) and pre-immune mouse IgG (panel d), which were used as negative controls. Importantly, the distribution of Jagged1 in mouse spleen coincided with that previously reported for c-Rel (Carrasco et al., 1994).

Immunofluorescence assays were performed on purified mouse splenic B cells to examine further the correlation between the expression of Jagged1 and c-Rel. Flow cytometry analysis demonstrated that spleen cells expressing the B-cell marker CD45R-B220 were also positive for c-Rel and for Jagged1 protein expression (Figure 4C). These results are consistent with our finding of a correlation between the expression of Rel/NF-κB factors and that of Jagged1. These data suggest that Jagged1 may play a role in B-cell activation, differentiation or function.

Discussion

The important role of the Rel and NF-κB factors in the immune system has elicited a fervent search for Rel-regulated genes that may participate in the control of cell proliferation, differentiation and malignant transformation (reviewed in Luque and Gelinas, 1997). Here, we report that the gene encoding the Notch ligand jagged1 is a novel NF-κB-responsive gene. The induction of jagged1 by endogenous NF-κB factors and its inhibition by a dominant IκBαΔN transgene demonstrate its dependence on nuclear NF-κB transcriptional activity. This study is also shows that c-Rel can trigger the Notch signaling pathway in neighboring cells. The endogenous expression of Jagged1 in splenic B cells is consistent with its Rel-dependent induction and points to a possible role for Jagged1 in B-cell activation, differentiation or function.

Interplay between the Rel/NF-κB and Notch signaling pathways

This study is the first to demonstrate that Rel/NF-κB can function upstream of Notch. In our assays, c-Rel, RelA and endogenous NF-κB factors elicited specific activation of the Notch ligand Jagged1. In turn, the expression of jagged1 correlated with the aggregation of non-adherent Jurkat T cells and Reh pre-B cells onto CCR43 cell monolayers. This effect was dependent on the transcriptional activity of c-Rel, as well as on the expression of an intact Notch receptor at the surface of co-cultivated lymphoid cells. Sup-T1 T cells that lacked a normal Notch1/TAN-1 allele and U266 B cells that did not express Notch1 failed to adhere to the cell monolayers and remained in suspension. We do not rule out the possibility that cytokines or adhesion molecules induced by c-Rel may also contribute to these interactions. However, the observed requirement for Notch receptor expression in these assays supports a model whereby c-Rel can promote
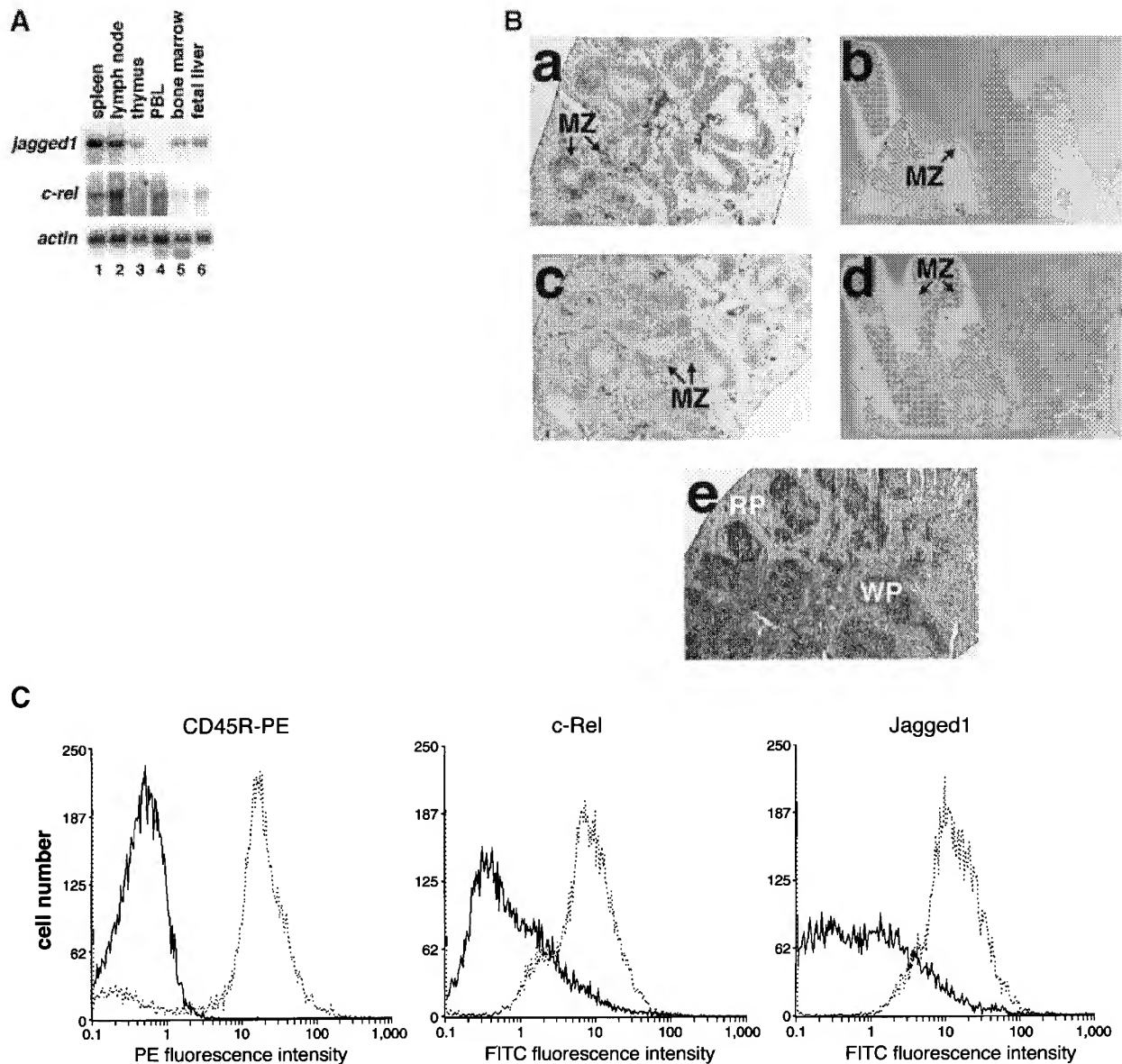
Fig. 4. Expression of *jagged1* in immune cells and tissues. (A) Northern blot analysis of endogenous *jagged1* gene expression in human immune tissues. A human immune system II Northern blot (Clontech) was hybridized to a human *jagged1* cDNA probe. The blot was rehybridized successively to c-*rel* and actin probes. (B) Immunohistochemical localization of Jagged1 to the B-cell areas of the spleen. Cross-sections from adult mouse spleen were analyzed by immunoperoxidase staining of a monoclonal anti-Jagged1 antibody (a) or a monoclonal anti-CD20 antibody (c). Normal rat serum (b) and pre-immune mouse IgG (d) were used as negative controls. The marginal zone (MZ) showed strong staining for Jagged1. Hematoxylin–eosin staining is shown (e). RP, red pulp; WP, white pulp. (C) Flow cytometry analysis of c-Rel and Jagged1 protein expression in mouse splenic B cells. Spleen cell populations were enriched for B cells by antibody plus complement-mediated killing of T cells. Cells were stained for expression of the B-cell marker CD45R-B220 with a PE-conjugated monoclonal antibody (left panel). This resulted in a population of >80% B cells. Cells were analyzed for expression of endogenous c-Rel (middle panel) or Jagged1 proteins (right panel) with polyclonal antibodies specific for c-Rel (sc-71) or Jagged1 (#PCR8), and a fluorescein isothiocyanate (FITC)-labeled secondary goat anti-rabbit antibody. Staining with the FITC-labeled secondary antibody alone was used a negative control (middle and right panels).

the functional interaction of Jagged1, with Notch receptors expressed at the surface of neighboring cells.

The Rel-dependent induction of *jagged1* in cell monolayers also coincided with the initiation of signaling downstream of Notch, as shown by the activation of two different CBF1/Su(H)/RBP-Jκ-regulated genes in co-cultivated lymphoid cells. Endogenous *HES-1* transcripts were upregulated reproducibly in Jurkat T cells cultured with CCR43 cell monolayers expressing c-Rel and Jagged1, or with HtTA-Jag10 cells expressing Jagged1

alone. This agrees with a recent report showing that ectopic expression of the vertebrate Notch ligand Delta-1 can transactivate endogenous *HES-1* gene expression in Notch1-expressing cells (Jarriault *et al.*, 1998). Likewise, the cultivation of Reh pre-B cells with a CCR43 cell monolayer led to the specific induction of mRNAs for the B-cell activation marker CD23. These results indicate that c-Rel can promote activation of the Notch signaling pathway and suggest that c-Rel-mediated activation of Jagged1 is responsible for this effect. Our findings do not

exclude the possibility that other transcription factors contribute to the regulation of *jagged1* gene expression in different cell lineages and at different stages of differentiation. It also remains to be determined whether Rel/NF-κB-mediated activation of *jagged1* gene expression is direct or indirect. Further work is required to isolate the *jagged1* promoter and establish whether its expression is controlled directly by Rel/NF-κB factors.

Relevance to the immune system

In addition to its role in neurogenesis, myogenesis, angiogenesis and retinal cell development, the Notch signaling pathway has also been implicated in hematopoiesis and in immune cell malignancies (reviewed in Robey, 1997). Consistent with this notion, the expression of Jagged1 was shown to promote the development of primitive hematopoietic precursor cells, whereas activated forms of Notch1 and Notch2 influenced the differentiation of myeloid progenitors in response to different cytokines (Bigas *et al.*, 1998; Jones *et al.*, 1998; Varnum-Finney *et al.*, 1998). In independent studies, overexpression of an activated form of Notch1 influenced T-cell differentiation during thymic development (Robey *et al.*, 1996; Washburn *et al.*, 1997). This process recently was proposed to involve the silencing of *CD4* gene expression by HES-1 (Kim and Siu, 1998).

The mapping of three human *Notch* genes to chromosomal locations associated with leukemia, lymphoma and myeloproliferative disorders has also suggested a role in immune cell proliferation and malignancy (Ellisen *et al.*, 1991; Larsson *et al.*, 1994; Milner *et al.*, 1994; Hasserjian *et al.*, 1996; Gridley, 1997). The demonstration that constitutively active forms of Notch1 induced T-cell leukemia/lymphoma in mice confirmed this prediction (Pear *et al.*, 1996). In experiments not shown here, we mapped the *jagged1* locus to human chromosome 20p12 by fluorescence *in situ* hybridization (FISH) in order to assess its possible implication in immune cell malignancies. While this study was in progress, mutations at this locus were shown to be associated with Alagille syndrome (Li *et al.*, 1997; Oda *et al.*, 1997). Although alterations in immune or hematopoietic function were not reported, it remains to be determined whether the disease results from haploinsufficiency or from a dominant-negative effect exerted by the mutant protein. The ability of the Notch signaling pathway to influence the differentiation and proliferation of different cell lineages may also depend on different inducing signals and on the cellular microenvironment.

Our immunohistochemistry analyses revealed that Jagged1 is highly expressed in the B-cell areas of the spleen, particularly in the splenic marginal zone that is rich in plasma and memory B cells. Consistent with these results, flow cytometry analyses further demonstrated a correlation between the expression of Jagged1 and c-Rel in purified mouse splenic B cells. Although the function of Jagged1 in secondary lymphoid organs remains to be determined, both Notch1 and Notch2 are also expressed in the spleen (Hsieh *et al.*, 1997; data not shown). This raises the possibility of a role for Jagged1-mediated signaling through Notch in the pathways that control the later stages of B-lymphocyte activation, differentiation and/or immune function. The ability of the EBNA-2

protein of Epstein–Barr virus to induce expression of the B-cell activation marker CD23 through its association with the Notch effector CBF1 in transformed B lymphocytes agrees with a role for Notch signaling in B cells (Ling *et al.*, 1994; Hsieh *et al.*, 1997). Our co-cultivation assays demonstrating that Jagged1-expressing cells induced *CD23* gene expression in neighboring B cells is consistent with this hypothesis. Future studies will help to define the role of Jagged1 in the splenic microenvironment, and to clarify whether it signals through Notch in the context of a heterotypic cell–cell interaction or in a cell-autonomous fashion. Recent work suggesting that soluble forms of Notch ligands can trigger signaling through Notch *in vivo* would be compatible with either possibility (Qi *et al.*, 1999).

c-*rel* knock-out mice are impaired for B-cell activation and antibody production (Kontgen *et al.*, 1995; Carrasco *et al.*, 1998). The observation that all known B-cell growth factors failed to rescue the proliferative defect of these cells suggests that c-Rel may regulate the expression of genes, other than those for cytokines and growth factors, which are crucial for the activation and proliferation of B lymphocytes (Kontgen *et al.*, 1995). Our finding that c-Rel can trigger the expression of Jagged1 raises the possibility of a connection between the Rel/NF-κB and Notch signaling pathways in secondary lymphoid organs. Thus, in addition to controlling the expression of cytokines, immunoregulatory and adhesion molecules, Rel/NF-κB factors may also trigger a Notch signaling cascade important for lymphocyte activation and immune function.

Materials and methods

Plasmids and cell culture

pUHD10-3 contained seven tetracycline operator sites upstream of a minimal cytomegalovirus (CMV) promoter (Gossen and Bujard, 1992). An *XhoI–EcoRI* fragment containing the human *jagged1* cDNA in pBluescript was subcloned into pUHD10-3, after addition of an *XbaI* linker to its *XhoI* end (pUHD10-3-*jagged1*). pHMR272 was used to confer resistance to the drug hygromycin B (Bernard *et al.*, 1985).

The parental HeLa-derived HtTA-1 cells stably expressed a fusion protein comprised of the *Escherichia coli* tetracycline repressor fused to the activation domain of the herpes simplex virus VP16 protein (tTA; Gossen and Bujard, 1992). HtTA-1-derived cell clones that conditionally expressed c-*rel* (CCR43 cells), *relA* (HtTA-RelA cells) or a truncated c-Rel protein lacking the c-*rel* transactivation domain were also described (CCR-H5 cells; Bash *et al.*, 1997; Zong *et al.*, 1998). The tetracycline-regulated HtTA-jag10 cell clone that conditionally expressed *jagged1* was generated by co-transfection of HtTA-1 cells with pUHD10-3-*jagged1* and pHMR272 using a modified calcium phosphate procedure, followed by selection for resistance to hygromycin B (225 U/ml, Calbiochem; Chen and Okayama, 1987). Drug-resistant colonies were screened for the inducible expression of Jagged1 using monoclonal antibody #TS1, specific for the intracellular domain of the human Jagged1 protein (a gift of Dr Artavanis-Tsakonas). All HtTA-1-derived cell clones were maintained in the presence of tetracycline (1 μg/ml).

Human Jurkat and Sup-T1 T-lymphoblastic leukemia cells, Reh pre-B cells and U266 B-myeloma cells were obtained from ATCC. The Jurkat T-cell clone constitutively expressing the IκBαΔN transdominant inhibitor of Rel/NF-κB was cultured in the presence of hygromycin B, as described previously (300 μg/ml; Chu *et al.*, 1997). Where indicated, Jurkat T cells (5×10^5/ml) were treated with PMA (50 ng/ml) plus ionomycin (1 μM) for 2 h to induce endogenous NF-κB/Rel activity. Cells were treated with dimethylsulfoxide (DMSO; 0.1%) as a control.

Cloning of a human jagged1 cDNA

Differential display PCR (DD-PCR) analysis was performed as described previously (Liang and Pardee, 1992) using RNA from uninduced and induced CCR43 cells. RNA samples (2 μg) were treated with DNase and reverse transcribed with Superscript reverse transcriptase (Gibco-

BRL) and a 5'-T$_{11}$CA primer. Partial cDNA libraries were PCR amplified with 5'-T$_{11}$CA and 5'-AGACGTCTGT-3' primers in the presence of [α-^{35}S]dATP and resolved on 6% sequencing gels. cDNA fragments differentially expressed in response to c-Rel were introduced into pCRII (Invitrogen) by TA cloning and screened by dot-blot analysis (Callard *et al.*, 1994). One of these cDNA fragments (clone 1-2, 106 bp) was used to screen a human spleen cDNA library in λgt11 (Clontech). Human clone λ2-3 contained a 1778 bp insert homologous to nucleotides 1059–2837 of the rat *jagged1* cDNA (DDBJ/EMBL/GenBank accession No. L38483). A full-length cDNA for human *jagged1* was isolated from a HeLa Uni-ZAP™ XR cDNA library (Stratagene) with probes derived from clone λ2-3 and from human expressed sequence tag (EST) clone 117734, homologous to the 3' end of rat *jagged1* (Research Genetics). Phages positive with both probes were excised in pBluescript phagemids. Human cDNA clone 14-6 contained a 5457 bp insert comprised of the complete coding region for *jagged1* (3657 bp) together with 263 bp of 5'-untranslated sequence and 1537 bp of 3'-untranslated sequence (DDBJ/EMBL/GenBank accession No. AF028593). The sequence of human *jagged1* cDNA clone 14-6 was analyzed using the EditSeq and MegAlign programs of DNASTAR.

Northern blot analysis

Total RNA (20 µg) extracted with RNAzol B (TEL-TEST) was analyzed in 1% agarose–formaldehyde gels and transferred onto Hybond-N or Hybond-NX membranes (Amersham). Membranes were baked for 10 min at 80°C and UV cross-linked with a Stratalinker (Stratagene). A multiple human immune system II Northern blot was purchased from Clontech. Probes were generated by random priming with Klenow DNA polymerase in the presence of ^{32}P-labeled dCTP and dGTP. Membranes were hybridized to probes specific for c-*rel*, *jagged1*, *delta1* (a gift of G.Weinmaster, UCLA, CA), IκBα (a gift of A.Baldwin, University of North Carolina, NC), *Notch1/TAN-1*, *HES-1* (a gift of R.Kageyama, Kyoto University, Japan), *CD23* (a gift of E.Kieff, Harvard Medical School, MA) or the actin gene. Where indicated, blots were hybridized to human Il-2Rα- and *gapdh*-specific probes.

Immunoblotting

Cell extracts were prepared and quantitated as described (Bash *et al.*, 1997). Proteins (20 µg) were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with immune serum, followed by donkey anti-rabbit (Amersham) or goat anti-rat horseradish peroxidase (HRP)-conjugated antibodies (Jackson Immuno-Research Labs, PA) and detected by enhanced chemiluminescence (Amersham). Rat monoclonal antibody #TS1 (a gift of S.Artavanis-Tsakonas, Yale University, CT) was specific for the intracellular domain of human Jagged1. Polyclonal anti-Rel antibody Ab #1801 specific for the unique C-terminus of c-Rel (Kumar and Gelinas, 1993) and an antibody specific for actin (Sigma) were also used.

Immunofluorescence and immunohistochemistry analyses

Cell surface expression of the human Jagged1 protein was detected by immunofluorescence of live non-permeabilized cells, with all steps prior to fixation carried out at 4°C. Cells were washed in phosphate-buffered saline (PBS), blocked in PBS plus 5% goat serum and incubated with polyclonal antibody #PCR8 raised against the extracellular domain of rat Jagged1 overnight (a gift of G.Weinmaster, UCLA, CA). Cells were washed in PBS, incubated with a biotinylated goat anti-rabbit antibody (Vector Labs) and then fixed in 4% paraformaldehyde. Cells were washed in PBS, stained with FITC-labeled streptavidin (Jackson Immuno-Research Labs) and mounted with 0.2% *p*-phenylenediamine (Sigma). Spleen cells from C57BL/6 mice were enriched by antibody plus complement-mediated cell killing with rat anti-mouse Thy-1.2 antibody J1j to deplete T cells. This resulted in a population of >80% B cells. Cells were analyzed by double-immunofluorescence staining for expression of the B220 B-cell marker with a phycoerythrin (PE)-conjugated rat monoclonal anti-mouse CD45R-B220 antibody (PharMingen), and for the expression of c-Rel or Jagged1 using rabbit polyclonal antibody sc-71 (c-Rel; Santa Cruz Biotechnology) or #PCR8 and a fluorescein-labeled goat anti-rabbit secondary antibody (Jackson ImmunoResearch Labs, PA). Immunohistochemistry analysis of cross-sections from adult mouse spleen was carried out by the Immunohistochemistry Core Facility (Cancer Institute of New Jersey, New Brunswick, NJ). Primary antibodies were rat monoclonal anti-Jagged1 antibody #TS1 and mouse monoclonal antibody #L26 that recognized the B-cell antigen CD20 (Dako Corporation). Normal rat serum and pre-immune mouse IgG were used as negative controls. Reactivity was revealed by immunoperoxidase staining.

Co-cultivation assays

HtTA-1, CCR43 or CCR-H5 cells were plated in duplicate in the presence of tetracycline. After 24 h, one dish from each series was induced in medium lacking tetracycline. After 48 h, the cells were washed three times with Dulbecco's modified Eagle's medium (DMEM) with or without tetracycline, and overlaid with Jurkat, Sup-T1, Reh or U266 non-adherent cells in suspension (2.5×10⁶ cells/dish). The co-cultures were incubated for 48 h. Photographs were taken after the cells remaining in suspension were washed away. For Northern blot analyses, firm tapping was used to dislodge the lymphoid cells that aggregated onto the adherent cell monolayer, prior to RNA extraction from the co-cultivated lymphoid cells.

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